THE EFFECTS OF DIETARY POLYUNSATURATED FATTY ACIDS ON PROSTATE CANCER-PROTEOMIC AND PHOSPHOPROTEOMIC STUDIES

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THE EFFECTS OF DIETARY POLYUNSATURATED FATTY ACIDS ON PROSTATE CANCER-PROTEOMIC AND PHOSPHOPROTEOMIC STUDIES

This dissertation studies the effects of fatty acids on prostate cancer. Prostate cancer is one of the most common malignant diseases in males in the U.S. Because of the slow progression of this disease, early intervention methods, especially, dietary fatty acid interventions are considered very important to control the disease in early stages. This study describes how the depletion of the enzyme for endogenous fatty acid synthesis, fatty acid synthase, influences the expression of enzymes that metabolize dietary fatty acids and show how dietary fatty acids affect prostate cancer protein expression and function. Fatty acid synthase is an oncoprotein overexpressed in prostate cancer and its expression is suppressed with omega-3 fatty acid treatment. This study finds that the depletion of fatty acid synthase by siRNA knockdown induces suppression of cyclooxygenase-2 and fatty acid desaturase-1. Our results also show that fish oil (omega-3 fatty acid), but not oleic acid (omega-9 fatty acid), suppresses prostate cancer cell viability. Assessment of fatty acid synthesis activity indicates that oleic acid is a more potent inhibitor than fish oil of *de novo* fatty acid biosynthesis. In addition, the inhibition of its activity occurs over several days while its effects on cell viability occur within 24 hours. To better understand this relationship, label free LC-MS/MS based mass spectrometry was carried out to determine global proteomic and phosphoproteomic profiles of the prostate cell line PC3, with longitudinal treatment with fish oil or oleic acid. With short-term fish oil treatment, sequestosome-1was elevated. Prolonged

treatment induced downregulation of microseminoprotein, a proinflammation factor, as well as proteins in the glycolysis pathway. In the phosphoproteomics study, we confidently identified 828 phosphopeptides from 361 phosphoproteins. Quantitative comparison between fish oil or oleic acid treated groups and the untreated group suggests that the fish oil induces changes in phosphorylation of proteins involved in the pathways associated with cell viability and metabolic processes, with fish oil inducing significant decreases in the levels of phospho-PDHA1^{Ser232} and phospho-PDHA1^{Ser300} and they were accompanied by an increase in PDH activity, suggesting a role for n-3 polyunsaturated fatty acids in controlling the balance between lipid and glucose oxidation.

Mu Wang, Ph.D., Chair

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AA	arachidonic acid
ABC	ammonium bicarbonate
ACN	acetonitrile
ALA	alpha-linolenic acid
AMF	autocrine motility factor
AR	androgen receptor
AUC	area under curve
BPH	benign prostatic hyperplasia
CE	collision energy
CM	conditioned medium
COX	cyclooxygenase
CQ	chloroquine
DHA	docosahexaenoic acid
DHT	dihydrotestosterone
DP	declustering potential
DRE	digital rectal examination
DTT	dithiothreitol
EPA	eicosapentaenoic acid
FA	fatty acid
FASN	fatty acid synthase
FDR	false discovery rate

FO	fish oil
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GO	gene ontology
GPI	glucose-6-phosphate isomerase
HETE	hydroxyicosatetraenoic acid
HODE	hydroxyoctadecadienoic acid
IPA	Ingenuity pathway analysis
LA	linoleic acid
LC	liquid chromatography
LOX	lipoxygenase
LTQ	linear ion-trap
MRM	Multi-Reaction-Monitoring
MS	mass spectrometry
MSMP	microseminoprotein
OA	oleic acid
PCa	prostate cancer
PCA	principle component analysis
PDH	pyruvate dehydrogenase
PG	prostaglandin
PGK1	phosphoglycerate kinase 1
PIA	proliferative inflammatory atrophy
PIN	prostatic intraepithelial neoplasia
PK	pyruvate kinase

PSA	prostate specific antigen
PTM	post-translational modification
PUFA	polyunsaturated fatty acids
QA	quality assurance
QC	quality control
SNPs	single nucleotide polymorphism
TFRC	transferrin receptor

Chapter 1 Introduction

1 Prostate function

The prostate, an important accessary organ in the male reproductive system, is a walnut size gland located very close to the bladder and weights around 30 grams. The prostate gland secretes prostatic fluid, composing about 30% of the final volume of seminal fluid. The urethra connects the bladder and the penis and passes through the center of the prostate. During ejaculation, when sperm travel from the testes and pass through the prostate, the muscle of the prostate contracts to secrete prostatic fluid, which forms semen with the sperm-containing fluid in urethra. The prostate consists of the peripheral zone, the central zone, and the transition zone and the epithelial cells of the gland are surrounded by the stroma. The peripheral zone occupies the major part of prostate and 70%-80% prostate cancer (PCa) occurs in this region. The function of prostate secretion is to liquefy semen and nourish sperm and by providing various elements, including proteolytic enzymes such as, prostate specific antigen (PSA) and ions including citrate and zinc. PSA belongs to the kallikrein-related peptidase family and functions to dissolve seminal coagulum and cervical mucus, allowing sperm to move freely to enter the uterus [1, 2]. Zinc is reported to help stabilize chromatin in sperm cells by maintaining the chromatin structure [3]. It is also known that high levels of zinc can increase the concentration of citrate [4], which is an important component of prostatic fluid. It is reported that concentration, transportation and metabolism of citrate are altered in PCa [5]. The prostate's function is very important to men's health and fertility. However, prostate disease is one of the most common health issues in men. For the

majority of men, prostatitis, benign prostatic hyperplasia (BPH), and/or prostate cancer, may develop some time during their lifetime.

2 Prostate cancer incidence and risk factors

PCa is currently the second most common cancer occurring in men [6] with an estimated 220,800 new cases diagnosed and 27,540 deaths in 2015 (NCI SEER Stat Fact Sheets) [7]. Death due to PCa accounts for 1%-2% of the total mortality in men. PCa related morbidity largely reduces quality of life by causing erectile dysfunction and urinary inconsistency. The risk of developing PCa is currently one in six men (16.7%) and the overall risk of death due to metastatic PCa is about 2.8% with the disease progressing relatively slowly to advanced stages [8] and in many cases, the disease develops very slowly and without symptoms [9]. Although, PCa occurrence is as high as 85% in men older than 80 years of age, it is rare in men under 40 [10].

Both endogenous and exogenous factors contribute to PCa risks. **Genetic influence:** Familial clustering is observed in PCa occurrence. Having close family members, such as father or brothers, developing prostate cancer, especially diagnosed before 65 years old, indicates a higher PCa risk [11], which suggests PCa is influenced genetically. Studies on susceptibility genes [12, 13] and single nucleotide polymorphisms (SNPs) [14] have been carried out and showed that these factors can only explain part of the familial risks. **Hormone influence:** Hormones, androgens [15, 16] and estrogens [17, 18], may affect PCa incidence but the study conclusions are inconsistent and controversial. **Inflammatory influence:** Whether chronic inflammation is etiologically related to PCa has also been studied. In some studies, men with history of

prostatitis and sexually transmitted diseases have been shown to have a higher PCa risk [19, 20], but some other studies show no association between transmitted sexual infections and PCa [21, 22]. Prostate inflammation and PCa may be linked as some molecules are found to be critical for both prostatitis and PCa development, including certain fatty acids, nutrients and proinflammatory cytokines [23, 24]. Proliferative inflammatory atrophy (PIA) in the prostate most often occurs in parallel with inflammation. PIA lesions are thought to develop with epithelial proliferation in response to inflammatory injury and are believed to be a potential precursor to adenocarcinoma [25].

Influence from environment and behavioral risk: Prostate risk also varies between ethnic populations such that African-American men have a much higher risk of developing PCa than Asians. Noticeably, Asian men living in the Unites States have lower PCa risk than Caucasians and African Americans but higher risk when compared to Asian men living in Asia [26]. These facts suggest PCa may be influenced by the combination of various factors and both genetic and dietary/environmental factors may contribute to cancer occurrence. Other factors, such as smoking [27], vasectomy [28], obesity [29], sexually transmitted disease [30] and inflammation [23] have also been studied but conclusions are inconsistent among the studies.

3 Prostate cancer diagnosis and treatment

PCa develops so slowly and many men with this disease have no symptoms when diagnosed. Screening helps patients be aware of the possible existence of PCa in early stages and screening is thought to decrease the number of men that are diagnosed with

PCa that has already progressed to advanced stages. Considering in many cases prostate cancer will remain indolent, patients with low risk PCa, or elderly patients with a family history of the disease, regular active surveillance is recommended to determine whether further treatment is necessary. PCa screening includes digital rectal examination (DRE) and PSA test. The prostate is located in front of the rectum and DRE detects unusual growth in the prostate. The PSA test is used to detect elevated serum protein levels, and PSA between 4ng/ml and 10ng/ml usually results in additional PCa surveillance and screening. In men diagnosed with PCa, a rapid rise in blood PSA levels may suggest disease progression or cancer relapse after treatment. However, controversies also exist concerning whether PSA screening is necessary. Although screening may help reduce the mortality caused by PCa by discovering the disease in early stages, many men that are screened still develop advanced PCa and die from it. Additionally, false positive results, or indolent tumors may never need intervention. Because of its poor predictive accuracy [31], PSA screening may result in over-diagnosis and unnecessary aggressive treatments, which may impair a patient's health and life.

A positive DRE combined with PSA elevation typically is followed by a prostate biopsy. Biopsy results are reported as Gleason scores. Pathologists categorize PCa tissue by five different patterns, ranging from well differentiated (pattern 1) to poorly differentiated (pattern 5) carcinoma cells. Gleason Scores are calculated by the sum of the numbers of primary and secondary common patterns (in some systems, primary and the highest grade). Other diagnostic parameters, such as PSA and DRE, and ultrasound will be combined with biopsy results to help to decide the next steps of treatment. Active surveillance is an option when the risk of treatment outweighs the risk of non-treatment,

especially when patients are physically weak and advanced in age, or there are no symptoms observed. Additionally, staging systems, which define whether the cancer has metastasized to other parts of the body, also guides the treatment decisions. Surgery, such as radical prostatectomy can be performed on stage T1 and T2 tumors, characterized by tumor growth limited to the gland and confined within the prostate capsule. For the majority of patients with localized PCa, surgery or radiation treatment is curative. However, in a subset of patients, the disease progresses to a more advanced stage, and becomes life threatening.

Hormone therapy may be used in patients with advanced prostate cancer, when tumor penetration is beyond the capsule, characterized by stage T3 and T4, or if cancer recurs after radiation or radical surgery, or when metastasis occurs (N1 stage, M1 stage, when tumors spread to lung, bone, lymph nodes, brain or other parts of the body) in combination with rapid PSA level increases.

Androgen is required for growth of the gland in almost every stage of prostate development. Testosterone and its more potent derivative dihydrotestosterone (DHT) binds to the androgen receptor (AR) and enters the nuclei to function as a transcription factor to regulate expression of multiple genes [32]. In most cases, PCa cell growth is also dependent on androgen stimulation, mainly induced by testosterone and DHT, however, high androgen serum levels are not positively associated with high PCa risk [16]. Hormone deprivation treatment can control PCa progression. Hormone therapies used to suppress the androgen pathway include surgical castration and chemical castration, such as luteinizing hormone-releasing hormone analogs, androgen-suppressing drugs and AR antagonists [33]. Besides these therapies, radiation therapy can also be

applied in some situations to minimize the tumor size. In some cases PCa can develop into more advanced stages and PCa cells can grow despite hormone blockade. Castration resistance may be attained through several mechanisms [34]: over expressed/ amplified AR [35, 36], hypersensitive or mutated AR [37], or constitutively active truncation [38] of AR gene/protein or over production of androgen in prostate cells [39]. PCa cells can also become castration-resistant when AR is over-activated by growth factor/cytokines [40], or some AR co-activators, like AIB1 [41] and ARA55 [42].

When PCa metastasizes to other organs and tissues and cancer cell growth becomes hormone-refractory, chemotherapy drugs, such as docetaxel and cabazitaxel, are most often used to extend patients' life and relieve some symptoms. However, in this stage of advanced PCa, especially when the metastasis is combined with castration resistance, PCa is usually incurable. To date no definitive factors have been associated with PCa development and progression and no curative therapy exists for advanced disease. The slow progression of the disease and the lethality of advanced cases make the prevention and control in early stages critically important. Given the long latency period of PCa progression coupled with the close association between incidence and aging, therapies that slow disease progression would help eliminate the need for surgery or radiation and prevent metastatic spread.

4 Fatty acid synthase (FASN) and prostate cancer

1) Expression and functions of FASN in cancer

Lipogenesis is considered to be both pro-inflammatory [43] and pro-oncogenic [44]. In many cancers, lipogenesis is increased and numerous studies suggest that a high-

fat diet enhances tumor risk [45]. Altered metabolism is a common feature in many cancers involving upregulated glycolysis [46], as well as elevated *de novo* lipid synthesis [47]. High lipogenesis equips cancers with survival advantages. Rapid cancer cell proliferation demands fast synthesis of cell membranes consisting of phospholipids, glycolipids, and cholesterol. Lipids through diet and cell synthesis can also provide a means of energy storage and cell signaling. Many pro-inflammatory and pro-oncogenic cytokines, and prostaglandins, are products of lipid metabolism [48]. The inhibition of lipogenesis and fatty acid (FA) degradation through beta-oxidation are targets for controlling cancer progression [49, 50].

FASN is a protein containing seven separate enzyme moieties that catalyze FA synthesis. It consists of two identical 272 kD multi-functional polypeptides and is the only protein in mammals responsible for *de novo* lipogenesis. FASN synthesizes palmitic acid by adding acetyl-CoA to malonyl-CoA to extend the FA chain by two carbons at a time to make a 16-carbon chain. Palmitic acid can then be utilized to further synthesize stearate and myristate. The expression level of FASN is found to be elevated in multiple cancers including melanoma [51], colorectal cancer [52], and PCa [53]. FASN is normally expressed at very low levels in the prostate due to high dietary fat intake but is up-regulated as early as PIN and is highly elevated in 82% of men with localized and metastatic PCa [54]. It has also been reported that an increased copy number of the FASN gene is often present in PCa tissue [55] and several germline SNPs of FASN are associated with higher PCa risk [56]. In addition, FASN is a cytoplasmic protein in normal cells. However, nuclear localization of FASN is associated with PCa progression [57]. In normal tissues, *de novo* synthesis of FAs through FASN is down-

regulated by the availability of dietary FAs [58]. Although elevated lipid content in tumor tissue was identified 80 years ago [59], the link between dietary and *de novo* fatty acids in PCa is still not clear. FASN is transcriptionally regulated by SREBP-1 [60], which is downstream of PI3K/Akt [61] and androgen/AR [62]. However, FASN expression remains elevated in the prostate tumors of men receiving androgen ablation therapy [54], indicating a loss of normal regulation of gene expression in cancer. In one PCa study, Nelfinavir, a drug for treatment of AIDs, down regulates FASN expression through suppression of the translocation of SREBP-1 [63]. One of our goals is to identify novel down-stream target(s) of FASN.

2) FASN expression and activity are associated with cancer progression

FASN is a metabolic oncoprotein and is being pursued as a therapeutic target [64]. Overexpression of FASN has been observed in many cancers and it promotes tumor progression through affecting oncogenic pathways [65, 66]. FASN inhibitors are being developed to decipher potential functions of FASN and help understand the underlying mechanisms with the ultimate goal of developing novel anti-tumor drugs against PCa [67].

High FASN expression renders cancer cells a greater survival advantage

High FASN levels have been found to trigger multiple tumorigenic pathways. For example, in pancreatic cancer, FASN activates EGFR/ERK pathway [65]. Likewise, in colorectal cancer, FASN overexpression increases cellular respiration [68]. Other examples include co-overexpression of FASN and phospho-c-met in papillary and follicular thyroid carcinomas [66]; and high co-expression of c-Met receptor kinase and

FASN in B-cell lymphoma [69]. One study also showed that FASN overexpression could induce Wnt1 palmitoylation, elevate Wnt1/beta-catenin signaling and promote tumor invasion in mice [70]. It has also been demonstrated that activation of the PI3K-Akt pathway can increase FASN expression [71], indicating FASN not only regulates down-stream oncogenic pathways but also be regulated by up-stream oncogenic pathways.

FASN inhibitors induce cell death and apoptosis

Previous studies on FASN utilized multiple FASN inhibitors to analyze the protein's function and its roles in pathway networks. These FASN inhibitors include Cerulenin, C75, luteolin, orlistat, TVB-3166, and several others. Cerulenin is a fungus derived natural compound and C75 is one of its synthetic analogs. Cerulenin and C75 are both found to induce apoptosis or suppress cell proliferation in breast cancer [72], melanoma [73], colorectal cancer [74], and PCa [75, 76]. Another natural compound, luteolin, is mainly derived from leaves of dietary vegetables, such as celery. As a FASN inhibitor, luteolin can inhibit the c-Met pathway [77] and importantly, it was found that adding palmitate could restore c-Met levels [37]. In addition, a similar study carried out in a breast cancer cell line, demonstrated that osthole, a fruit-derived compound was reported to suppress FASN in breast cancer cells overexpressing HER2 [78]. Osthole can suppress c-met, which was confirmed with C75 treatment and again c-Met suppression can be reversed by palmitate [38]. Another FASN inhibitor orlistat, originally used to treat obesity, inhibits proliferation, as well as metastasis in oral squamous cell carcinomas [79] and suppresses cell proliferation of PCa cell line PC3 both *in vitro* and *in vivo* [80]. Furthermore, a recent study using a newly developed FASN inhibitor, TVB-3166,

showed growth suppression in many cell lines from ovarian, pancreatic, prostate, and hematopoietic cancers through multiple pathways including modulating lipid raft structure, inhibiting Akt and beta-catenin signaling, as well as the sterol synthesis pathway [81].

5 Dietary fatty acid consumption and prostate cancer

1) Dietary nutrition and prostate cancer

Epidemiology studies, clinical trials and many *in vitro* studies have revealed an increased risk of developing PCa and a worse prognosis after treatment is associated with lifestyle, and the diet is a major part of it. Many studies employing dietary agents for modulation of cancer initiation and progression have been carried out. For example, pomegranate juice was found to inhibit PCa cell growth [82]. One effective component of pomegranate juice is ellagic acid (EA), which triggers an apoptosis pathway and suppresses cyclin D1 and cdk [83]. A study using a mouse model of PCa indicated that curcumin slows down PCa growth, and AR expression is decreased in the curcumin treated group [84]. Green tea is also considered to have anti-tumor activity through the epigallocatechin gallate (ECGC) component of the tea acting in part via inhibition of FASN. A meta-analysis on low grade PCa showed that tea consumption is protective regardless the type of the tea [85]. One study also showed that coffee consumption may decrease PCa risk [86], but another study showed that the risk reduction occurs only when consuming boiled coffee [87]. Intake of Vitamin C reduces PCa risk [88], Vitamin D inhibits PCa proliferation when combined with metformin [89] or Vitamin A [90]. In contrast, vitamin K antagonist intake decreases PCa risk [91, 92]. In prostate tumors, the

main energy source appears to be through lipid pathways [93], thus FA metabolism may play a critical and specific role in PCa progression.

2) An overview of dietary fatty acid

Daily dietary FA consumption consists of saturated fat and polyunsaturated fatty acids (PUFAs). Most PUFAs consumed can be divided into sub-categories of n-3, n-6 and n-9 PUFAs based on the position of the first double bond counting from the methyl end. In most cooking oils, oleic acid (OA, C18: 1), which is monounsaturated fatty acid, is the major form of dietary n-9 fatty acids. While mammalian cells can interconvert the PUFAs within each of the n-3/6 series, the two series are not interchangeable. In addition, neither n-6 nor n-3 PUFAs can be synthesized *de novo* in mammals, thus dietary intake is critical for daily demands. The most abundant PUFAs in daily cooking oil are n-6 FAs, including linoleic acid (LA) (18:2) and arachidonic acid (AA) (20:4). Besides flaxseed oil (mostly alpha-linolenic acid, ALA, 18:3), n-3 FAs exist in very small amounts in other vegetable oils. One of the important animal sources of FAs is fish oil (FO; salmon, mackerel, tuna and other cold water marine fish). FO contains n-3 PUFAs with longer carbon chains than ALA: eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6). Compared to saturated FAs, PUFAs are correlated with lower risk of cardiovascular disease [94]. However, the ratio of different n-3, n-6 and n-9 PUFAs consumed will impact health benefits as some PUFAs compete as substrates to trigger anti-tumorigenic or pro-tumorigenic pathways [95].

3) Impact of dietary fatty acid on prostate cancer

The risks and benefits of dietary FA composition, and the ratio of n-3/n-6 PUFAs in particular, are considered to be associated with the prevention and control of various diseases, especially PCa [95]. Consumption of n-6 PUFAs including LA and AA from certain plant oils correlates with increased PCa risk whereas consumption of n-3 FAs found in marine fish, EPA and DHA, correlates with decreased risk [96].

Clinical trials and population studies have been carried out to investigate the correlation between different dietary FA consumption and PCa. A study involving 6,272 Swedish men found that men who do not eat fish have a much higher frequency of developing PCa compared to their fish-consuming counterparts [97]. A recent clinical study on low-risk PCa patients receiving a high n-3 PUFA diet intervention found that the increase of EPA in prostate tissue decreases the cancer progression risk [98]. Interestingly, this paper found prostate cancer risk is neither significantly associated with EPA intake nor with EPA level in red blood cells [98]. Patients receiving low-fat FO diet demonstrated a lower cell cycle progression score and lower 15-Hydroxyicosatetraenoic acid (HETE), a pro-inflammatory metabolite [99]. Similarly, a phase-II clinical trial showed that patients receiving low fat and low n-6/n-3 PUFA ratio diets have a lower cancer progression index compared to the western style diet group [100]. A nested case-control study of multiple different cancers showed that in patients with a lower intake of n-3 PUFA, soluble intercellular adhesion molecule-1 (sICAM-1) levels were associated with cancer risk while in patients with a higher n-3 PUFA intake, there is no such association [101].

Another study on circulating FAs supports the preventive function of n-3 PUFAs [102] and in this study they measured erythrocyte n-3 PUFA levels. However, a prospective research study in PCa patients with localized tumors indicated that long chain n-3 PUFAs in plasma phospholipid fatty acids were associated with higher PCa risk; and to the contrary, n-6 PUFAs were correlated with lower PCa risk [103]. This finding is consistent with two other studies, in which n-3 phospholipid in plasma or serum was measured [104, 105]. One of the studies found trans-fatty acids were associated with lower PCa risk, while DHA was associated with higher high-grade PCa risk [105]. This plasma phospholipid study generated controversy and points to a gap in understanding the FO mechanism of action, cell lipid uptake, tissue-specific FA concentrations and lipid metabolism [106, 107]. All of these factors vary widely from one individual to another and the reasons behind the opposing findings on n-3 fatty acids and cancer risk remain indeterminate.

N-3 PUFAs have also been shown to modulate FA synthesis through inhibition of the FASN expression [108]. Several studies have shown that n-3 PUFAs suppresses Akt, EGF and AR pathways [109-111]. Activation of these pathways can directly or indirectly promote changes in FASN levels by activating its transcription factor SREBP-C1.

In addition to n-3 and n-6 FAs, other FAs such as OA may also play important roles in reducing cancer risk. Unlike n-3 PUFAs, which are derived from limited sources (flaxseed, certain fish), n-9 fatty acids (such as OA) are one of the dominant components in most daily cooking oil. Results from studies investigating OA and cancer, however, are controversial. Some studies indicate OA has inhibitory effects on PCa cell proliferation [112], while others observed a higher OA/stearic ratio in untreated and more

advanced PCa patients [113, 114]. However, whether the high level of OA/stearic ratio is due to more endogenous OA production, dietary OA consumption, or the decreased level of stearic acid remains to be investigated. In the present study, we selected OA, a commonly consumed FA, as one of the controls to be used in comparison with FO to identify the pathways activated or suppressed in PCa cells, specifically by FO.

Another role of n-3 PUFAs may be in sensitization of PCa, as well as other cancers to chemotherapy or other stress-inducing factors. It is reported that n-3 PUFAs restored chemosensitivity of colon cancer cells against multiple drugs [115] and mammary tumors against docetaxel [116]. However, there was also a study that showed an opposite effect with combination treatment of n-3 PUFA and tamoxifen which increased Erk1/2 and Akt phosphorylation and tampered apoptosis induced by tamoxifen alone [117]. A study in leukemia cells also showed n-3 PUFAs can activate RAS/ERK/C/EBPb pathway through DNA demethylation [118]. Although several large studies in patients have reached different conclusions regarding n-3 PUFA intake and PCa risk [103, 119], most *in vitro* and *in vivo* studies have demonstrated that n-3 PUFA exerts inhibitory functions on PCa cells. For example, EPA was found to inhibit the phosphorylation of ERK, FAK, and p70S6K [120] and EPA induced JNK phosphorylation and apoptosis [121]. Similarly, it is reported that both n-3 and n-6 PUFAs suppress JAK1, STAT1, ERK1/2, and JNK phosphorylation induced by IFN-gamma [122]. Another study also found n-3 PUFAs suppress phosphorylation of Akt but have no effect on phospho- or total Erk 1/2 [123]. Additional studies have shown that DHA suppressed phosphorylation at AKT (T308) but changed AKT (S473) phosphorylated protein localization without affecting its phosphorylation level, as well as

regulated Akt's interaction with BAD [124]. An earlier study indicated the suppression of PDK/Akt/Bad is SDC-1 dependent and induced apoptosis [109]. One of the suggested causes of changes to signaling molecule function is modulation of phosphorylation by disruptions of lipid raft microdomains by n-3 PUFA [125]. Lipid raft microdomains harbor numerous signal transduction proteins. In addition to the classic signaling pathways, such as Akt, JNK or Erk, which were studied widely in cancer research, there are may be other undiscovered pathways modulated by n-3 PUFA. The regulation of cancer cells by different FAs involves multiple pathways, networks as well as numerous proteins and small molecules that impact cell survival, stress response and metabolism.

6 Goal of the study

As the diet provides daily exposure to FAs, there is an extended capacity to exert influence on numerous cellular events including energy storage and breakdown, cell membrane structure, subcellular trafficking, and bioactive metabolite synthesis. Many of these pathways can be affected by different FA moieties [126]. To gain a clearer picture of the changes induced by FAs, a quantitative proteomics approach may help elucidate changes in global protein expression and post-translational modifications. This state-of-the-art approach has become an effective approach to identify factors involved in FA metabolism [127].

We suggest that the understanding of the regulatory effects of FO discovered from previous studies is far from complete. Our overall hypothesis is that FO exerts anti-oncogenic functions in prostate cancer cells through altering expression and post-translational modifications of multiple proteins. Our aim is to better understand the

molecular targets and key pathways of FO metabolism. To achieve this, two large-scale proteomic studies were performed, one to assess global protein expression changes and the other to delineate global protein phosphorylation changes induced by fatty acid treatments.

Chapter 2 Dietary fatty acid metabolizing enzymes regulated by FASN

1 Preliminary data and significance of the study

As an oncoprotein, FASN, is a candidate therapeutic target for cancer [64]. To investigate how FASN affects PCa cells and identify potential molecular targets of FASN, a series of siFASN knockdown experiments were carried out using multiple PCa cell lines and microarray analyses. As shown in **Fig. 2-1**, the expression levels of several genes involved in PUFA metabolism were significantly altered when PPC-1 cells were treated with siFASN. Microarray results from the LNCaP cell line also showed similar gene expression patterns.

Genes affected by FASN knockdown include choline kinase, phospholipase C, Δ-5 (FADS1) and Δ-6 (FADS2) desaturases, thromboxane A synthase, and cyclooxygenase-2 (COX2). Choline kinase catalyzes the phosphorylation of choline to form phosphocholine. Overexpression of this gene has been demonstrated to confer oncogenic properties in kidney cells while high enzyme activity correlates with tumor grade in breast carcinoma [128, 129]. In this study, this enzyme is down-regulated. Similarly in gene expression profile, phospholipase C, which liberates arachidonic acid (AA) from membrane phospholipids, as well as Δ-5 (FADS1) and Δ-6 (FADS2) desaturases, which both participate in the metabolism of dietary n-3 and n-6 fatty acids to EPA and AA, respectively [130], are down-regulated. Δ-6 desaturase also plays a role in the further metabolism of AA and EPA. In addition, Δ-9 desaturase was down-regulated by FASN depletion and is involved in the conversion of stearic acid to OA [130]. Thromboxane A synthase, which is responsible for catalyzing prostaglandin H2 to thromboxane A2 [131], was also down-regulated. Finally, a small degree reduction in

COX2 mRNA was induced with FASN knockdown. In addition to this study, a previous immunohistochemistry study showed an over-expression of 15-lipoxygenase-1 (15-LOX1) in prostate tumor tissue as compared with normal adjacent tissue [132].

COX and LOX are two families of enzymes that can catalyze reactions between PUFAs and oxygen to produce eicosenoids. COX generates prostanoids and LOX generates leukotrienes. Both the two enzymes have several forms. Human 15-LOX1 is a highly-regulated, tissue- and cell-type-specific lipid-peroxidating enzyme that has several functions ranging from physiological membrane remodeling to pathogenesis of atherosclerosis, inflammation, and carcinogenesis [133]. 15-LOX1 metabolizes LA to 13(S)-hydroxyoctadecadienoic acid [13-(S)-HODE], which can regulate cell growth, differentiation and vascular homeostasis [134-143]. A previous study demonstrated that an n-3 diet in a PCa xenograft mouse model decreased serum PSA levels, induced apoptosis, and inhibited tumor proliferation [144]. The same study demonstrated that EPA competed with LA as a substrate for the lipoxygenase (15-LOX1), resulting in a decrease in the pro-tumorigenic metabolite 13(S)-HODE, and an increase in 15-HEPE primarily associated with anti-inflammatory and anti-tumorigenic effects.

In addition to 15-LOX1, evidence also suggests that other metabolic enzymes such as COX2 play important roles in PCa pathobiology. AA also acts as a substrate for cyclooxygenases COX1 and COX2. Most tissues constitutively express low levels of COX1 with minimal or no COX2. Studies support an association between inflammation and PCa [145, 146]. Growth factors or inflammatory agents rapidly induce COX2 expression in the prostate [147] and studies show that COX2 is overexpressed in PCa [148-150]. COX2 overexpression, leading to the production of pro-inflammatory PGs

(e.g., PGE_2), possibly contributes to PCa pathobiology. EPA also competes with AA for COX2 activity [151]. Although the metabolites of PUFAs directly impact PCa through both diet and metabolic enzyme expression, the mechanisms of n-3 PUFA action remain to be determined. In this study we sought to find out whether FASN depletion will lead to alteration of expression of these enzymes, as microarray data suggested.

2 Materials and methods

1) Cell culture

The PCa cell line, PC3 (ATCC), was grown in RPMI-160 medium (HyClone, Logan, UT, USA) supplemented with 10% FBS (Sigma-Aldrich, St. Louis, MO, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Lonza, Walkersville, MD, USA) in 37°C with 5% CO_2. For the AR positive PCa cell line, LAPC4, IMDM medium (HyClone, Logan, UT, USA) was used.

2) siRNA transfection

Cells were plated at 30~60 $x10^4$ cells/well in 6-well plates. Within 24 hrs post-plating, cells were transfected with synthesized siRNA targeting FASN. The siRNA-free, transfection reagent-only control, and the non-targeting siRNA sequence were used as negative controls, referred as *control* and *siNonT*, respectively. Transfection kits were purchased from Pharmacon RNAi Technologies (Denver, CO, USA) and the siRNA transfection was performed according to the protocol.

3) Western blot

Cell pellets were lysed with NP-40 lysis buffer supplemented with 1% protease inhibitor (Thermo-Fisher Scientific, Waltham, MA) and phosphatase inhibitor (Sigma-Aldrich, St. Louis, MO, USA). Laemmli buffer (Bio-Rad, Hercules, CA, USA) containing 5% 2-mercaptoethonal (Fisher Scientific, Pittsburgh, PA, USA) was added to cell lysate supernatant and the mixtures were boiled to denature. Proteins were separated by SDS-PAGE gel then transferred to PVDF membrane (Bio-Rad, Hercules, CA, USA). Blocking was done in 5% BSA (Roche, Indianapolis, IN, USA). PVDF membrane was incubated with primary antibodies against COX2 (Cell Signaling Technology, MA, USA). Bands were detected by ECL™ Western Blotting Detection Reagents (Amersham™, GE Healthcare, Piscataway, NJ, USA).

4) Quantitative mRNA measurement

Cells were harvested and RNA extraction was carried out using RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA). Reverse transcription was performed using QuantiTect Reverse Transcription kit (Qiagen). Realtime PCR was performed on QuantStudio 12K Flex (Applied Biosystems, Foster City, CA, USA) and the protocol was designed according to Taqman Gene Expression Assay (Applied Biosystems, Foster City, CA, USA). The gene of interest was labeled with reporter dye FAM and Glucuronidase β (GUSB), which serves as internal standard was labeled with reporter dye VIC. Realtime PCR primers and probes targeting FASN (assay ID: Hs01005622_m1), COX2 (assay ID: Hs00153133_m1), FADS1 (assay ID: Hs00203685_m1), 15LOX1 (assay ID: Hs00993765_g1), SCD (assay ID: Hs01682761_m1), FADS2 (assay ID: Hs00188654_m1), TBXAS1 (assay ID: Hs01022706_m1), GUSB (assay ID:

Hs00939627_m1), as well as Mastermix (the reaction solution), were designed and purchased from Applied Biosystems.

5) Liquid chromatography/mass spectrometry (LC/MS)-based unbiased global proteomic analysis

The cell pellets were lysed and sonicated in 8M urea. Bradford assay was carried out to determine the total protein concentration. Chicken lysozyme, which serves as a QA/QC reference protein was spiked in every sample to be analyzed (0.6%, w/w). Reduction and alkylation were carried out with triethylphophine and iodoethanol at 37 °C. The resulting samples were dried overnight in a speed vacuum and re-dissolved in 70 μl of 100 mM ammonium bicarbonate (ABC) buffer. Tryptic digestion was carried out by adding trypsin (0.6% of sample protein, w/w) (Worthington, Lakewood, NJ) diluted in 100 mM ABC buffer and letting the samples to be digested overnight. All the samples were filtered by a 0.45 μm PVDF filter (Emd Millipore Corporation, Billerica, MA) prior to each injection. Duplicate injections were performed for every sample in a randomized order for all 36 injections using a 20-μg total protein per injection. The tryptic peptides were loaded onto a C18 microbore column (Xbridge 2mm* 50mm, Waters, Milford, MA) and eluted with a gradient from 5% to 40% acetonitrile and 0.1% formic acid over 155 min at a flow rate of 200 μl/min. Data was acquired on a Thermo-Fisher Scientific linear ion-trap (LTQ) mass spectrometer. "Triple-play" mode was applied for data acquisition, which consists of MS scan, Zoom scan, and MS/MS scan. SEQUEST® search engine was used for database search against International Protein Index (IPI) database (version v3.83). Mass tolerances of precursor ions and product ions were set at 2.0 amu and 1.0 amu, respectively. Protein/peptide identification validations were carried out using

PeptideProphet/ProteinProphet software developed by Institute of Systems Biology (ISB) and available for free downloading. Label-free protein quantification was performed using an in-house developed "ProteinQuantXL" software [152]. A one-way ANOVA analysis was performed on the data (n=6) and q-values were calculated.

6) Pathway and statistical analyses

The gene ontology networks were extracted using Ingenuity pathway analysis software (IPA). Associated network functions were extracted from differentially expressed genes (also known as "focus genes"). The network generation was carried out as follows: 1) according to the ranking of interconnectedness of these genes, top ranked genes are selected; 2) The most connected gene is selected and the rest of the focus genes are added to this seed gene; 3) If the network still contains less than 35 genes, genes outside the focus gene list are added to the network if they are related to the network; 4) p-value is defined as the probability of finding numbers of *f* or more focus genes in the network in a randomly selected gene list (e.g., N=35); and 5) Score = $-\log_{10}$ (p-value). A lower p-value (= a higher score) indicates a less likelihood that the network extraction is random [153].

Canonical pathway analysis determines the association between proteins of interest and certain canonical pathways. The lower the p-value, the less likely that the association is random. It also gives a ratio representing the overlap of the gene list of interest and the gene list of certain canonical pathways [154].

Unpaired t-test analysis was performed using a statistic software Graphpad 6.0. q-value was calculated using a web-based tool: *q-value estimation for false discovery rate (FDR) control* (http://qvalue.princeton.edu)

3 Results

1) Validation of candidate target of FASN

Validation of microarray results by Western Blot was performed using an additional siRNA knockdown system (Pharmacon RNA Technologies). Western blot analysis showed that the FASN protein levels were significantly reduced 72 hours post-transfection **(Fig. 2-2 A)**. In the FASN activity assay, the pathway activity was reduced by approximately 30% over control and non-targeting siRNA treated cells **(Fig. 2-2 B).** The genes for validation were selected from the microarray results and genes known to be involved in functional pathways of PUFA processing were examined by Real-time PCR. In PC3 cells, COX2 mRNA levels showed a significant decrease (p=0.0009) in the siFASN treated group 48 hours post-transfection **(Fig. 2-3 A)** but after 72 hours, the observed mRNA change is not significant (P=0.075) **(Fig. 2-3 B)**. The COX2 protein levels were also decreased in siFASN transfected cells compared to control and non-targeting siRNA treated cells **(Fig. 2-3 E)**. In addition to COX2, FADS1 levels were also significantly suppressed (p=0.049) 48 hours post-transfection **(Fig. 2-3 A)**; although no significant change was observed after 72 hrs **(Fig. 2-3 B)**, 15LOX1 levels appeared to have a decreasing trend in expression with FASN siRNA treatment but the effect was not found to be significant **(Fig. 2-3 B)**. Since PC3 is an AR negative PCa cell line and represents hormone insensitivity, we also tested FASN knockdown in a PCa cell line with wild-type AR, LAPC4 **(Fig. 2-3 C)**. LAPC4 has very low COX2 mRNA levels compared to PC3 (12 cycle difference in Real-time-PCR). However, COX2 mRNA levels were significantly decreased (p=0.002) 72 hours post-transfection rather than 48

hours as observed in the PC3 cells **(Fig. 2-3 D)**. FADS1 and 15LOX1, which decrease with FASN knock down in PC3 cells, were not altered in LAPC4.

2) Proteomics analysis of siFASN knockdown

To investigate the downstream pathways of FASN, an LC/MS-based label-free global protein quantification technology was used to compare the proteomes of PCa cells affected by FASN. 1187 proteins were identified with peptide probability over 0.8 and protein probability over 0.9. Probability was generated by protein/peptide identification using PeptideProphet/ProteinProphet software. Among them, 516 (43.5%) showed differential expressions among the groups according to ANOVA $p \leq 0.05$ (n=6) and $q \leq 0.05$ thresholds. When comparing siFASN and siNonT groups, 201 out of 516 proteins (39.0%) (besides FASN) are found significantly changed (multiple comparison test $p \leq 0.05$, fold change $\geq |1.2|$).

Noticeably, the fold change of FASN in this proteomic study is smaller than in Q-PCR and western blots with only 1.6-fold change between the siFASN and the siNonT groups and 1.8-fold change compared to control. However, protein frequency (protein frequency=number of injections in which protein X was detected/total injection number) for all injections shows that in the siFASN-treated group, the protein frequency is 8.3%, comparing to 91.7% in control group and 100% in siNonT group, which indicates a substantial knockdown of FASN.

Ingenuity Pathway Analysis (IPA) was applied to extract biological networks from the 201 proteins that were differentially regulated. Top scored networks [Score = $-\log_{10}$ (p-value)] were extracted as follows **(Table 2-1 A):** 1) amino acid metabolism, post-translational modification, small molecule biochemistry; 2) hematological disease,

organismal injury and abnormalities, cell morphology; 3) cell death and survival, DNA replication, recombination, and repair, energy production; 4) cellular movement, cell morphology, connective tissue development and function; and 5) carbohydrate metabolism, small molecule biochemistry, developmental disorder. The main molecular and cellular functions include: cell death and survival, cellular growth and proliferation, nucleic acid metabolism, carbohydrate metabolism, small molecule biochemistry. Top canonical pathways were also generated **(Table 2-1 B),** they are: gluconeogenesis I, glycolysis I, epithelial adherens junction signaling, EIF2 signaling and integrin signaling.

Among these 201 proteins, 18 proteins have significant (multiple comparison $P \leq 0.05$) differences between control and siFASN groups. Control and siNonT groups demonstrated the same trend compared to the siFASN group. Sixteen of the proteins were annotated in IPA **(Table 2-2).** While a 9-kDa protein encoded by transferrin receptor (TFRC) was down-regulated in the siFASN group, the other 15 proteins were found to have increased expression levels with FASN knockdown. The 7 upstream regulators of these proteins are: ethionine, indomethacin, lipopolysaccharide CD3, IL1B, MYC3, and IL15 **(Fig. 2-4).**

4 Discussion

Metabolites of n-6 and n-3 PUFAs directly impact PCa tumor development and the ability to do so depends on both diet and the levels of enzymes responsible for metabolizing these PUFAs. A study in liver has shown that the COX inhibitor celecoxib can down-regulate FASN independent of COX2 [155], while our results revealed the possibility of a reverse regulation: that COX2 can be regulated by FASN. COX2, being a

non-constitutive COX, (also known as prostaglandin-endoperoxide synthase (PTGS)), can convert AA to PGE2, an inflammatory molecule, which plays a crucial role in prostate inflammation and carcinogenesis. Both COX2 and FASN were found extensively expressed in PCa [55, 156]. The positive correlation between COX2 and FASN was also reported in Barrett's esophagus (intestinal metaplasia), which is a risk factor for esophageal adenocarcinoma [157]. Both enzymes have been found to be down-regulated by FO or specific n-3 PUFAs [108, 158]. In a trial in which PCa patients received an n-3 rich enriched diet, 4 of 7 patients showed decreasing COX2 mRNA expression in prostatic tissue [158]. COX2 expression levels were decreased in the group with the lower n-6/n-3 ratio *in vivo* and *in vitro* [159]. In addition, inhibitors of FASN or COX2 are potent tumor suppressors [76, 160]. COX2 inhibitors have been widely studied in the treatment of PCa [161, 162]. The relationship or interactions between FASN and COX2 are not well documented. The results of our study showed that there is a possible positive regulation of FASN on COX2. This might suggest that FASN mediates the effects of n-3 or n-6 PUFA on COX2, the increase of which is not only a potential trigger, but also one of consequences of PCa progression. The roles n-6 PUFA and COX2 play may vary among different cancer types. Apart from the mitogenic aspect of FASN, our result suggest that the FASN-COX2 pathway might play an important part in inflammation at early stage disease and angiogenesis in the later progressive stage. The combination of DHA and the COX2 inhibitor celecoxib enhances suppression of proliferation and induction of apoptosis in PCa cell lines significantly more than either treatment alone [160]. However, some conflicting reports have been published in which a phase II low fat/fish oil diet in PCa patients demonstrated that a 4-6 week dietary

intervention prior to radical prostatectomy suppressed the tumor cell proliferation without changing COX2 or PGE2 levels [100].

In our current study, we found that in addition to COX2, FADS1 mRNA level decreased with FASN knockdown in PC3 cells while FADS2 did not change. The mRNA level of 15LOX1 also showed an insignificant decreasing trend when FASN was knocked down. FADS1 and FADS2 are the enzymes involved in long chain n-6/n-3 synthesis. Gene variants of these two enzymes influence PUFA composition in blood and breast milk [163], and affect the individual PUFA change in response to FO [164, 165]. Our results suggest that FADS1 might be affected by the change of FAs synthesis. In regards to PCa, however, a recent report showed contradictory results that FADS1 in periprostatic adipose tissue was down-regulated in obese/overweight PCa patients [166]. With respect to the role of 15LOX1, a mouse model demonstrated that 15LOX1-expressing mice are more likely to have prostatic intraepithelial neoplasia (PIN) [167]. There is also evidence that the metabolites of DHA under 15LOX1 can act as anti-proliferation factors [168], illustrating the competitive effects of n-3 over n-6 PUFA for 15LOX1. These support the assumption that 15LOX1 might mediate FASN's effect on PCa progression. Therefore n-3 may function in several ways including decreasing tissue concentrations of AA, so there is less available to form n-6 eicosanoids, competing with AA for COX and LOX enzymes or potentially counteracting their AA derived metabolites.

Our proteomics study and the follow-up IPA analysis identified an additional 16 potential targets of FASN. These proteins are significantly changed under siFASN treatment condition when compared to control and siNonTargeting groups, respectively.

In the up-regulated proteins identified, annexin-A1 has been reported to play an important role in inducing apoptosis of PCa cells [169, 170]. However, expression of lysophosphatidylcholine acyltransferase 1 (LPCAT1) was reported to correlate with higher risk of PCa [171, 172], as well as TPD52L2, cDNA FLJ53635, highly similar to Homo sapiens tumor protein D52-like 2 (TPD52L2), transcript variant 6, mRNA, which predicts PCa tumor progression after radical prostatectomy when combined with squalene epoxidase (SQLE) [173]. In addition, talin-1 (TLN1), which is expressed at higher levels in PCa than in BPH [174]; and S100A11, which also is associated with PCa development [175], were both down-regulated with FASN knockdown. TFRC, which is down-regulated with siFASN, was reported to be up-regulated under treatment with C75, a FASN inhibitor [176]. FASN is a target of n-3 PUFA as well as a regulator of other proteins and pathways downstream. The knockdown of FASN induces alterations in multiple downstream targets. In addition, as shown in results of FASN expression and activity, a dramatic decrease in FASN expression does not necessarily lead to complete suppression in FASN activity, which is believed to be due to the long half-life of FASN. In the proteomic study, it is noticeable in the proteomic study that most changes in protein levels are very minor, but statistically significant. One explanation is that since the remaining FASN in the siFASN group still has considerable activity, the impact of the depletion of FASN on the biological processes might be limited, especially at a protein expression level; another explanation is that altered FASN level and activity change protein post-translational modifications. As a highly expressed oncoprotein, FASN has become an attractive therapeutic target and understanding FASN inhibition in tumors by n-3 PUFAs provides further insight into the mechanism of PCa initiation and

progression. In addition this study reveals links to how FO impacts the FA molecular pathways of PCa.

Table 2-1 Network and pathway analysis of differentially expressed proteins in FASN depleted PCa cells

(A) Top associated Network Functions (Score = $-\log_{10}$ (p-value))

ID	Associated Network Functions	Score
1	Amino Acid Metabolism, Post-Translational Modification, Small Molecule Biochemistry	62
2	Hematological Disease, Organismal Injury and Abnormalities, Cell Morphology	45
3	Cell Death and Survival, DNA Replication, Recombination, and Repair, Energy Production	41
4	Cellular Movement, Cell Morphology, Connective Tissue Development and Function	40
5	Carbohydrate Metabolism, Small Molecule Biochemistry, Developmental Disorder	34

(B) Top Canonical Pathways

Name	p-value	ratio
Gluconeogenesis I	4.31E-10	7/47 (0.149)
Glycolysis I	6.04E-10	7/41 (0.171)
Epithelial Adherens Junction Signaling	3.04E-07	10/154 (0.065)
EIF2 Signaling	2.53E-06	10/201 (0.05)
Integrin Signaling	4.66E-06	10/208 (0.048)

Table 2-2 Differentially expressed proteins in FASN depleted PC3 cells

(C: control; F: siFASN; N: siNonT)

Protein ID	Gene Name	Protein Name	FC_F_C	Multiple_Comparison_p_F_C	FC_N_C	Multiple_Comparison_p_N_C	FC_N_F	Multiple_Comparison_p_N_F	ANOVA_p_value
IPI00005859	KRT75	Keratin, type II cytoskeletal 75	1.3	0.004	-1.1	0.2	-1.4	0.004	0.0005
IPI00013895	S100A11	Protein S100-A11	1.2	0.006	-1.1	0.008	-1.3	0.0001	2.00E-05
IPI01013714	CAPZB	Capping protein (Actin filament) muscle Z-line, beta	1.1	0.006	-1.2	0.003	-1.3	0.0002	1.00E-05
IPI00942539	ACTN4	39 kDa protein	1.2	0.01	-1.1	0.03	-1.3	0.003	0.0003
IPI00000816	YWHAE	Isoform 1 of 14-3-3 protein epsilon	1.1	0.01	-1.1	0.1	-1.2	0.007	0.002
IPI00022202	SLC25A3	Isoform A of Phosphate carrier protein, mitochondrial	1.2	0.02	-1.2	0.01	-1.4	0.001	0.0002
IPI00643231	ANXA1	Annexin A1	1.2	0.02	-1.2	0.02	-1.4	0.0009	0.0002
IPI00171626	LPCAT1	Lysophosphatidylcholine acyltransferase 1	1.1	0.02	-1.1	0.02	-1.3	0.0007	0.0001
IPI00215777	SLC25A3	Isoform B of Phosphate carrier protein, mitochondrial	1.1	0.02	-1.2	0.01	-1.3	0.0009	0.0001
IPI00005159	ACTR2	Actin-related protein 2	1.1	0.02	-1.1	0.02	-1.2	0.0003	6.00E-05
IPI00878551	P4HB	cDNA FLJ59430, highly similar to Protein disulfide-isomerase	1.1	0.02	-1.1	0.1	-1.2	0.002	0.0007
IPI00910487	SERPINH1	cDNA FLJ52569, highly similar to Collagen-binding protein 2	1.2	0.02	1	0.7	-1.2	0.03	0.005
IPI00926534	ZYX	Protein	1.2	0.02	-1	0.5	-1.2	0.02	0.005
IPI00925162	TFRC	9 kDa protein	-1.2	0.02	1.1	0.2	1.3	0.02	0.005
IPI00743469	TPD52L2	cDNA FLJ53635, highly similar to Homo sapiens tumor	1.1	0.04	-1.1	0.02	-1.3	0.004	0.0005

		protein D52-like 2 (TPD52L2), transcript variant 6, mRNA							
IPI00298994	TLN1	Talin-1	1.2	0.05	-1.1	0.1	-1.3	0.007	0.002

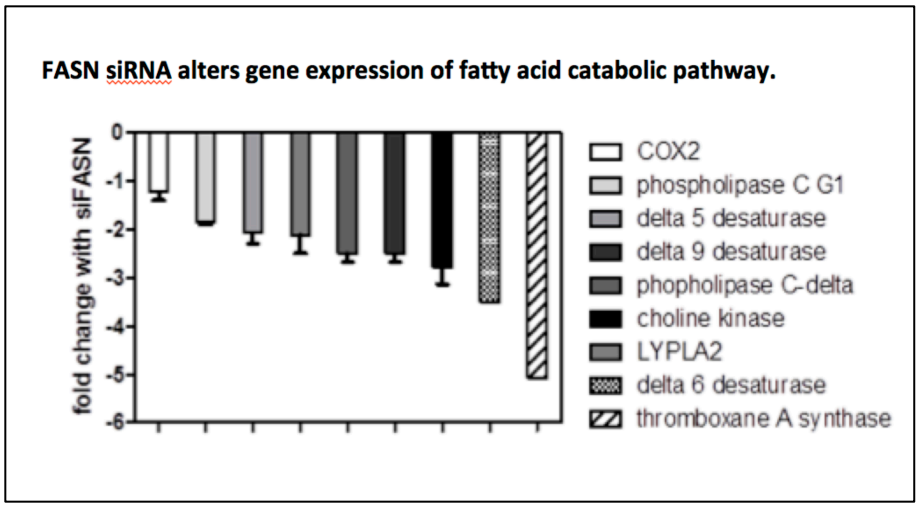
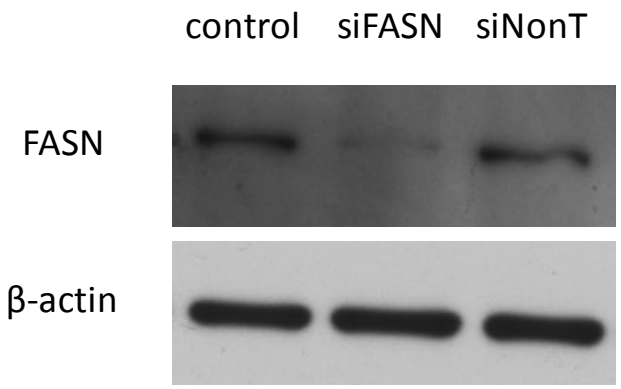


Figure 2-1 FASN siRNA alters gene expression of fatty acid pathways.

A gene array analysis of FASN siRNA treated PPC1 using Affymetrix U131 chip identified genes involved in phosphatidylcholine synthesis (choline kinase), arachadonic acid liberation (phospholipases) and AA metabolism (Δ5- and Δ6-desaturases), prostaglandin synthesis (COX2), Δ9-desaturase was also downregulated by FASN siRNA and is involved in the conversion of stearic to oleic acid.

A



B

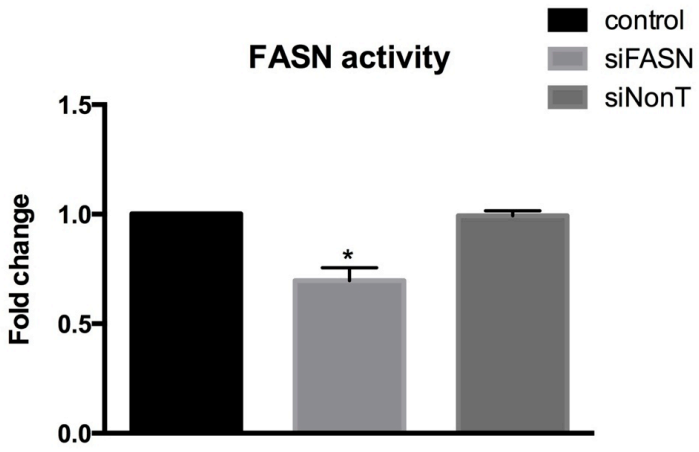


Figure 2-2 FASN was knocked down by siRNA in PC3 cells.

A: FASN protein level by Western Blot 72 hours post transfection. **B**: FASN activity level 72 hours post transfection. Labeled fatty acids were obtained by ^{14}C-acetate incorporation and Folch extraction. DPM values were measured by scintillation count. Fold change was calculated by normalizing results to control group. (unpaired t test comparing siFASN and siNonT group, n=3, *: $p<0.05$)

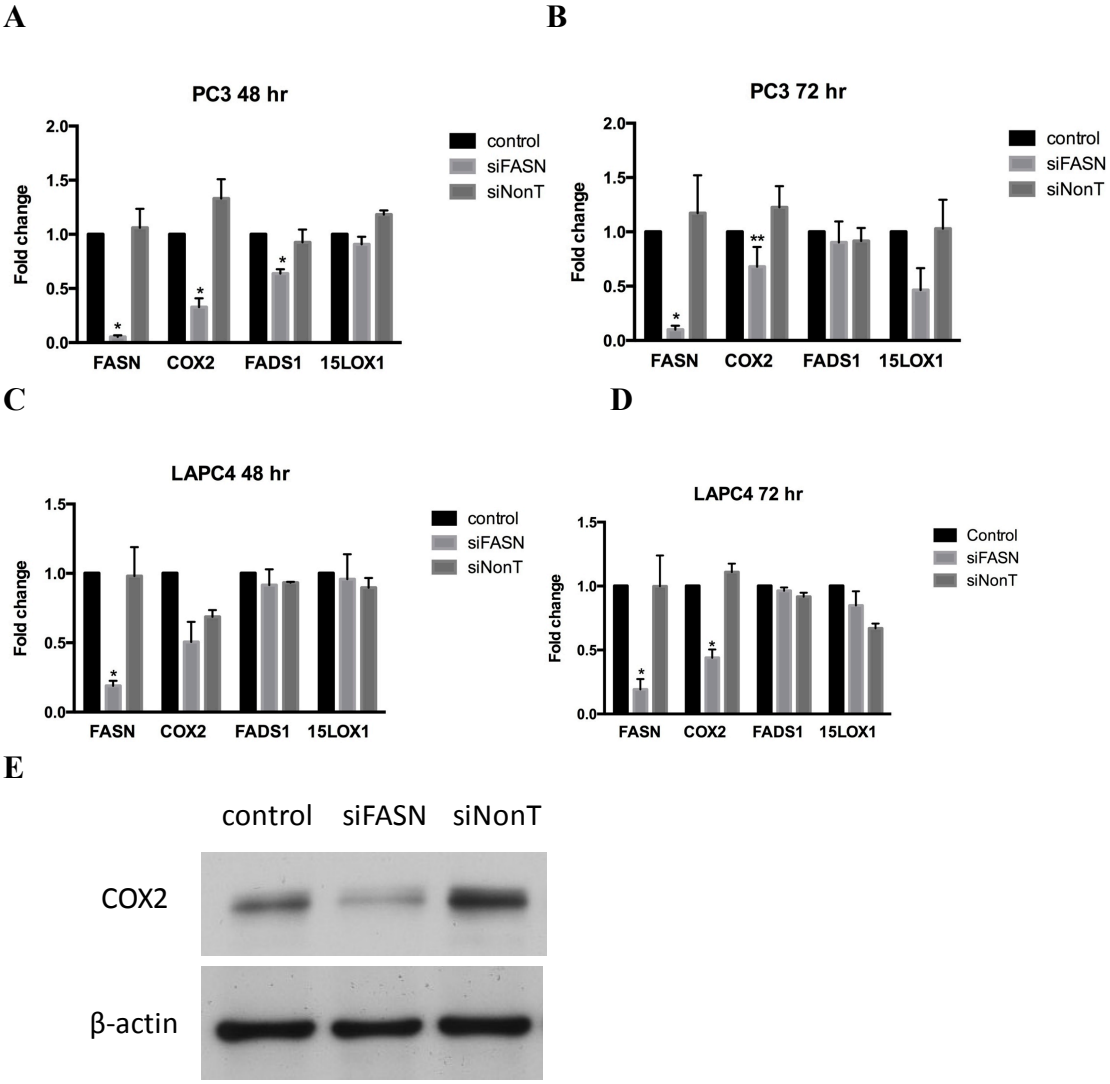


Figure 2-3 FASN knockdown changes the expression of PUFA metabolic enzymes.

A-B: mRNA levels of FASN, CO2, FADS1 and 15LOX1 in PC3 cell were measured by realtime-PCR 48 hours and 72 hours post transfection. n=5, **C-D:** mRNA levels of FASN, CO2, FADS1 and 15LOX1 in LAPC4 cell were measured by realtime-PCR 48 hours and 72 hours post transfection. n=3 **E:** COX2 protein level by Western Blot 72 hours post transfection in PC3 cells. (unpaired t test comparing siFASN and siNonT group, *: $P<0.05$. ** $P<0.1$)

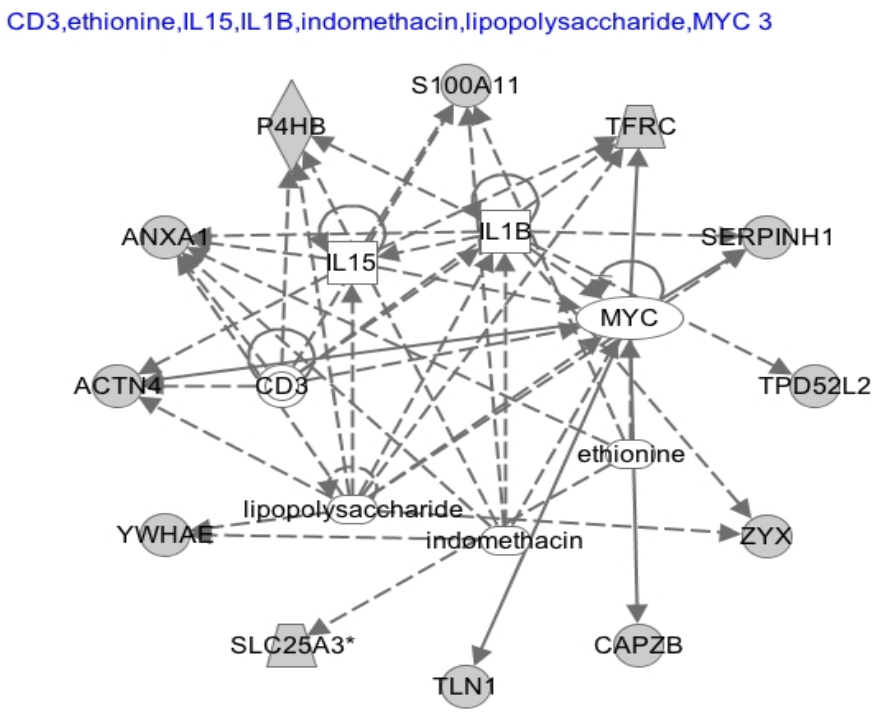


Figure 2-4 IPA based upstream regulator analysis on 16 proteins differentialy expressed in siFASN group compared to control and siNonTargeting group.

The top 7 upstream-regulators (IL15, IL1B, MYC, CD3, lipopolysaccharide, indomethacin and ehionine) shown in the inner circle modulate 12 of these 16 proteins, which are shown in outer circle. Solid line: direct regulation; Dash line: indirect regulation.

Chapter 3 Global proteome alterations induced by polyunsaturated fatty acids in PCa cells

1 Introduction

Except for flaxseed oil (alpha-linolenic acid, ALA) and some fish oil (DHA and EPA), n-3 PUFA, one of the essential FAs, exist as a very small portion of dietary oil intake. To the contrary, saturated fatty acids, OA, and n-6 PUFAs are main components of daily cooking oil, including corn, peanut or sunflower seed oil. Proteomic studies of their effects on human health have been previously carried out [177-183]. However, most of these studies used two-dimensional gel electrophoresis approaches with study objectives varying from insulin resistance to colon inflammation [177-183]. Large-scale proteomic studies on dietary fish oil effects on PCa using the LC/MS/MS based label-free protein quantification method has not yet been reported.

The aim of this study was to better understand the pathways involved in FO inhibition of PCa cell viability and to further investigate FO and OA's effects in global protein expression. We compared global protein expression profiles of PC3 cells treated with FO and OA at two different time points. Protein expression data presented here clearly demonstrate that FA-mediated molecular changes are not directly linked to FASN activity and the function of FAs may likely be mediated through multiple targets.

2 Materials and methods

1) Cell culture

The PCa cell line, PC3 (ATCC), was grown in RPMI-160 medium (HyClone, Logan, UT, USA) supplemented with 10% FBS (Sigma-Aldrich, St. Louis, MO, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Lonza, Walkersville, MD, USA) in 37°C with 5% CO_2. For the AR positive PCa cell line, LAPC4, IMDM medium (HyClone, Logan, UT, USA) was used.

2) Fatty acid treatment

FO and OA were purchased from KD Pharma Bexbach GmbH (Bexbach, Germany). FO capsules contained 90% n-3 fatty acid ethyl ester (40% EPA, 40% DHA and 10% other n-3 PUFAs). OA capsules contained 90% oleic acid ethyl ester. 100 mM stock was made by dissolving fatty acids (FAs) in ethanol. When treating the cells, FAs-ethanol stock solution was mixed into complete medium at a 1:1000 ratio to a final concentration of 100 μM, while 0.1% ethanol was used in controls. For day-6 treatment samples, the medium was changed four days after the treatment with a fresh medium containing FAs. To harvest conditioned medium (CM), the growth medium was replace with serum-free medium, CM was harvested 24 hrs later and cell debris was removed with centrifugation at 1,500 rpm. CM volume was normalized based on cell count with fresh serum-free medium to reach a same volume/cell number ratio among different treatments.

3) Chloroquine (CQ) treatment

In autophagy suppression assays, chloroquine diphosphate salt (Sigma-Aldrich, St. Louis, MO, USA) was diluted to 8 μM in medium.

4) MTT and colony formation assay

Cells were seeded in 96-well plates (1,000 PC3 cells or 6,700 LAPC4 cells per well, respectively) and grown in complete medium with different FA treatments. The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay was carried out by incubating cells with MTT (Sigma-Aldrich, St. Louis, MO, USA) for 4 hrs. DMSO was used to dissolve the formazan. Absorbance was measured at 570 nm. Cells were seeded in 6-well plates (1,000 PC3 cells or 3,000 LAPC4 cells per well, respectively) and grown in complete medium with different FA treatments. Colonies formed at approximately 1 week for PC3 cells and 1.5 weeks for LAPC4 cells. Colonies were fixed and stained with crystal violet, washed and counted.

5) Western blot

Cell pellets were lysed in NP-40 lysis buffer supplemented with 1% protease inhibitor (Thermo-Fisher Scientific, Waltham, MA) and phosphatase inhibitor (Sigma-Aldrich, St. Louis, MO, USA). Laemmli buffer (Bio-Rad, Hercules, CA, USA) containing 5% 2-mercaptoethonal (Fisher Scientific, Pittsburgh, PA, USA) was added to cell lysate supernatant and the mixtures were boiled to denature. Proteins were separated by SDS-PAGE gel then transferred to PVDF membrane (Bio-Rad, Hercules, CA, USA). Blocking was carried out in 5% BSA (Roche, Indianapolis, IN, USA). PVDF membrane was incubated with primary antibodies anti-MSMP (Abnova, Walnut, CA, USA), anti-sequestosome-1 (Novus Biologicals, Littleton, CO, USA), anti-beta-actin (Fisher

Scientific, Pittsburgh, PA, USA), anti-phospho-PDHA1 (Ser232) (LifeSpan Biosciences, Seattle, WA) and anti-alpha-tubulin (Sigma-Aldrich, St. Louis, MO) and secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and Cell Signaling Technology (Danvers, MA, USA) sequentially. Bands were detected by ECL™ Western Blotting Detection Reagents (Amersham™, GE Healthcare, Piscataway, NJ, USA). Quantification was carried out using ImageJ, an open source image-processing program.

6) Quantitative mRNA measurement

Cells were harvested and RNA extraction was carried out using RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA). Reverse transcription was performed using QuantiTect Reverse Transcription kit (Qiagen). Realtime PCR was performed on QuantStudio 12K Flex (Applied Biosystems, Foster City, CA, USA) and the protocol was designed according to Taqman Gene Expression Assay (Applied Biosystems, Foster City, CA, USA). The gene of interest was labeled with reporter dye FAM and Glucuronidase β (GUSB), which serves as internal standard and was labeled with reporter dye VIC. Realtime PCR primers and probes targeting SQSTM1 (assay ID Hs01061917_g1 and GUSB (assay ID: Hs00939627_m1), as well as Mastermix (the reaction solution), were designed and purchased from Applied Biosystems.

7) Fatty acid synthesis activity

Cells were incubated with complete culture medium with 1 μCi/ml 1,2-^{14}C-acetic acid sodium salt (PerkinElmer, Waltham, MA) was added to complete medium and cells were incubated for 2 hrs. The medium was discarded. For lipid extraction, 0.9 ml chloroform/methanol (2:1, v/v) mixture and 0.7 ml 4 mM $MgCl_2$ were added to the cells. The mixture was vortexed thoroughly and centrifuged at 10,000 rpm. The upper aqueous

layer was removed and the lipid was maintained in bottom layer, which is the chloroform fraction. Extraction was repeated for 3 times. Every sample was air dried in fume hood, then re-dissolved in 200 µl chloroform and finally transferred to 10 ml scintillation fluid Ultima Gold™ F (PerkinElmer, Waltham, MA). Disintegration Per Minute (DPM) value was measured by TRI-CARB 2100TR liquid scintillation analyzer (Packard Instrument Company, Kennesaw, GA).

8) Label-free quantitative global proteomic study

For global unbiased proteomic analysis, cell pellets were lysed in 8 M urea and 10 mM dithiothreitol (DTT) and then sonicated on ice. Protein concentrations were measured by BCA protein assay. Lysates with same amount of proteins were taken from each sample and chicken lysozyme was spiked into each sample at a final concentration of 0.5% (w/w), serving as a quality assurance (QA)/quality control (QC) reference. Reduction/alkylation of cysteine residues was carried out by incubating the cell extracts with acetonitrile (ACN)/iodoethanol/triethylphosphine (195:4:1, v/v/v) mixture. Samples were dried in a speed vacuum concentrator (Genevac, NY, USA) and resuspended in 310 µl of 100 mM ammonium bicarbonate, followed by overnight trypsin (Worthington, Lakewood, NJ) digestion (trypsin:protein=1:25). Desalting was carried out by repeating ACN-H_2O with 0.1% formic acid wash in Silica C18 MacroSpin Column (The Nest Group, Southborough, MA, USA). The resulting peptides were eluted by 80% ACN in last step and speed-vac dried. Samples were resuspended in 5% ACN in water with 0.1% formic acid and filtered through Ultrafree-MC Centrifugal filters (EMD Millipore, Billerica, MA). Peptides were eluted with a linear gradient from 5% to 80% ACN developed over 120 min at a flow rate of 50 µl/min, and effluent was electro-sprayed into

the LTQ Orbitrap Velos Pro mass spectrometer (Thermo-Fisher Scientific, Waltham, MA, USA). Each sample was analyzed by two injections and total injections were carried out in a randomly arranged sequence. X!tandem algorithms were applied to search the acquired data against UniProt human database (released in April 2013). Mass tolerance of precursor ion and product ion were 10 ppm and ± 1.0 Da, respectively. Peptides and proteins were validated using PeptideProphet and ProteinProphet in the Trans-Proteomics Pipeline (TPP, v.4.6). All the proteins listed in this study have protein probability >90% and peptide probability >80%. Label-free quantification was carried out using IdentiQuantXL^{TM} [152].

9) Multi-Reaction-Monitoring (MRM) experiment

Sample preparation, including cell lysis, reduction/alkylation and digestion was carried out the same way as that in proteomic discovery. Mass spectrometric analyses were performed on an AB/SCIEX 4000 Qtrap mass spectrometer (Sciex, Framingham, MA, USA) interfaced with a Dionex U-3000 UHPLC system (Thermo-Fisher Scientific, Waltham, MA, USA). Peptides were injected on an ODS-100V C18 column (1.0mm x 150mm, Tosoh Biosciences, South San Francisco, CA, USA) and eluted with a linear gradient from 5 to 80% acetonitrile over 32 min at a flow rate of 50 µl/min. The parameters were as follows: curtain gas of 40.0 psi, collision gas of 7 psi, ion spray voltage of 5500 V, temperature set at 500 ^{o}C, ion source gas 1 of 50.0 psi, ion source gas 2 of 40.0 psi. Entrance potential is set on 10.0 V; collision cell exit potential is 12.0 V. Data acquisition was carried out using the AB/SCIEX Analyst® software. For MRM development, the Skyline software was first used to generate candidate peptides and theoretical transitions for each peptide. PC3 cell lysate was tested to select the best

experimental peptide and transitions, which could be stably detected and also had high peak intensity. One specific transition was monitored for each target peptide, which represents one specific protein. Each monitored transition was confirmed by spiking in varied concentration of the corresponding synthetic peptides **(Fig. 3-1 & Fig. 3-2)**. For FSCN1, a transition for target peptide ^{23}YLTAEAFGFK^{32} was monitored: m/z 573.8 ($M+2H^+$) → m/z 870.4. For ITGB, a transition for target peptide ^{182}IGFGSFVEK^{190} was monitored: m/z 492.3 ($M+2H^+$) → m/z 666.3. For CANX, a transition for target peptide ^{78}GTLSGWILSK^{87} was monitored: m/z 531.3 ($M+2H^+$) → m/z 790.4. For MSMP, a transition for target peptide ^{52}YFTLGESWLR^{61} was monitored: m/z 636.3 ($M+2H^+$) → m/z 747.4. For SCP2, a transition for target peptide ^{48}IGGIFAFK^{55} was monitored: m/z 426.8 ($M+2H^+$) → m/z 739.4. A transition for chicken lysozyme, which serves as external standard, target peptide ^{40}GYSLGNWVCAAK^{61} was monitored: m/z 656.8 ($M+2H^+$) → m/z 892.4. We also monitored a transition for RPL6, which serves as the internal standard, target peptide: ^{211}HLTDAYFK^{218}, m/z 497.8 ($M+2H^+$) → m/z 744.4. Declustering potential (DP) and collision energy (CE) were optimized for each peptide. Scan time for each peptide was 120 msec and total scan time was 0.875 sec. Quantification of target peptide was performed using Skyline software to calculate area under curve (AUC) of extracted ion chromatograms.

10) Proteolytic activity measurement by DQ-BSA

PC3 cells were plated on 40 mm diameter glass bottom plates at a density of 40,000 cells/dish. Twenty-four hours post treatment of FAs, DQ-BSA was added to medium reaching a final concentration of 10 μg/ml. After 1 hour of incubation, the medium was changed to regular FAs containing but DQ-BSA free complete medium.

After another 2 hours incubation, cells were washed by PBS and fixed in 2% PFA for 10 min. Cell images were captured using ZEISS fluorescent microscope through AxioVision Rel 4.8 software. Laser in Channel of Rhodamine was used to excite BODIPY® dye to emit red fluorescence.

11) Biostatistic and bioinformatic analyses

The gene ontology networks were extracted using Ingenuity pathway analysis (IPA) (refer to Chapter 2-2-5)). Basic statistics (t-test, ANOVA and q-value) analyses were performed using Graphpad 6.0, Excel and R based q-value analysis website (http://qvalue.princeton.edu). Principle component analysis (PCA) was performed on the raw data set of protein abundance and a biplot was generated to show abundance variations and outliers if there are any [184]. PCA was performed using XLSTAT in Excel [185].

3 Results:

1) FO, not OA inhibits PCa cell growth

A MTT cell viability assay was performed to assess cell metabolic activity upon PUFA treatment. Shown in **Fig. 3-3 A**, FO, not OA, suppresses cell viability. A significant difference in cell growth (FO=74.8%C, $p=0.0105$) was observed after 24 hours in the FO treated cells but not in OA group (OA=101.4%C, $p=0.5493$). This observation was confirmed by clonogenic assays, in which colony density was significantly lower with FO treatment (**Fig. 3-3 B**). FO treatment demonstrated significant inhibition of colony formation ($p<0.05$) with no LAPC4 colonies observed in

FO treated group after 1.5 weeks. Again, the OA group showed no significant change as compared to control group ($p=0.2842$). During colony formation, vacuoles were observed in the FO treated cells, which remained single cells and stopped proliferating (**Fig. 3-3 C**). Clonogenic cell survival assays were also performed on androgen receptor (AR) positive LAPC4 cells. Potent inhibition of colony formation by FO was also observed in the LAPC4 cells (**Fig. 3-3 D**).

2) Both FO and OA inhibit FA synthesis and OA is a more potent inhibitor than FO

De novo FA synthesis was measured in the FO and OA treated groups after three days and six days treatment. At day three there was no change in fatty acid synthesis with fish oil treatment (**Fig. 3-4 A**), while a 30.97% ($p=0.0001$) suppression was observed in the OA group. After six days of treatment, a significant suppression of FA synthesis was observed in both FO (27.11%, $p<0.05$) and OA (49.21%, $p<0.0001$) groups (**Fig. 3-4 B**). Additionally, OA's FA synthesis level was lower than with FO treatment (OA=69.7%FO, $p=0.0301$), which suggests that OA is a more potent FA synthesis inhibitor than FO. This observation also implicates that the viability inhibition effect of FO on PC3 cells may not be directly linked to FA synthesis.

3) The differential proteome profile in prostate cancer cells treated with FO or OA

LC-MS-based label-free protein quantification was performed to investigate the longitudinal regulation of FO and OA on PCa cells. PC3 cells were plated at ~170,000 cells/plate on 100-mm plates with the complete medium with 0.1% ethanol, 100 μM FO, or 100 μM OA, respectively. Cells were harvested on day 1 and day 6 after treatment for MS analysis. A total of 1,478 proteins were identified with protein probability >99.8% and peptide probability >80%. Protein and peptide probabilities were produced through

calculation by TPP. Random sequences were set up serving as decoy sequences. A protein identification probability >99.8% corresponds to a FDR of <1%, which is a commonly used cutoff in the proteomics field.

Protein expression comparison was conducted based on treatment conditions (control, FO, and OA) and time-course (day-1 and day-6). Three hundred and twelve proteins were positively identified based on p-values less than 0.05. As indicated in the **Table 3-1**, majority of the technical variations are under 10% with a few outliers based on the internal control (chicken lysozyme) data and the overall %CV is below 13% **(Table 3-1)**. This is what we expected from this type of global protein profiling experiment with the platform we applied. The data was further filtered by FDR-adjusted p value (q value cut off=0.05) and the expression levels of 127 proteins were significantly changed upon treatment **(Table 3-2)**. Principal component analysis (PCA) was performed on this 127-protein dataset that consisted of 6 treatment groups with 5 replicates in each group. Prolonged FO treated group (FO-6 day) was a distinct group indicated by the red circle, separated from the other treatments (**Fig. 3-5 A**). The blue and yellow circles indicate there were fewer protein changes among treatments in day-1 and between control and OA group in day-6. By comparing each FO or OA group to its same-day corresponding control group, 73 proteins showed significant changes ($p \leq 0.05$). Log_2(Ratio<FO/C>) and Log_2(Ratio<OA/C>) values of protein entries were clustered by one minus Pearson Correlation (Gene E online analysis platform, http://www.broadinstitute.org/cancer/software/GENE-E/). Three major clusters were generated (shown in **Fig. 3-5 B**). Cluster A contained 7 proteins (encoded by 6 genes) (**Table 3-3**), including SCP2 and acyl-coenzyme A oxidase which participate in fatty acid

transport and metabolism [186, 187]; coactosin-like protein is reported to be involved in PUFA metabolism [188]. Cluster B showed that five of the six proteins (encoded by 4 genes) were significantly elevated in the FO group one day after treatment, and protein expression levels were higher than the OA group. Among these proteins, sequestosome-1 (SQSTM1) and heat shock 70 kDa protein 1A/1B (HSPA1A) were identified and have roles in the autophagy process [189, 190]. In cluster C, which consisted of 60 protein entries, no significant changes were observed in FO and OA groups in the first day of treatment. However, on day-6, FO-treated and OA-treated groups showed divergent protein expression changes. In the FO-treated group, 53 out of 60 proteins showed a significant decrease in expression while in the OA-treated group, the majority of differentially regulated proteins were elevated and eight of the changes were significant. Ingenuity® pathway analysis (IPA) indicated that cell movement, growth and proliferation, cell development, cell death/survival, and carbohydrate metabolism were the most significant molecular and cellular functions involved (**Table 3-4**), while glycolysis and gluconeogenesis were the top canonical pathways**.** Four enzymes from cluster C: phosphoglycerate kinase 1 (PGK1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), pyruvate kinase isozymes M1/M2 (PKM) and its isoform M1 (PK) and glucose-6-phosphate isomerase (GPI) were all significantly decreased in expression in FO-6-day treated group, but have little change in the OA-6-day treated group (**Fig. 3-6**).

In combination with IPA, the proteins with significant expression changes were selected based on the following criteria: 1) protein probability >99.8%; 2) two or more unique peptide sequences identified with high confidence; 3) within every group,

ANOVA p-value ≤ 0.05, q-value ≤ 0.05; 4) multiple comparison test p-value ≤ 5/ $N_{total\ tests}$-; 5) protein frequency (occurrence in all injections) >50%; 6) association with cancer, prostate, or lipid processing; 7) absolute fold change >1.3. Under these stringent criteria, we identified only one differentially regulated protein, SQSTM-1, in the day-1 treatment group. SQSTM-1 was elevated (1.7-1.8 fold) in the FO group compared to control and OA group. In the 6-day treatment comparison, the expression levels of 4 proteins were significantly altered as compared to the controls (**Table 3-5**).

4) Key differentially expressed protein validation

Both Western blot and MRM-based methods were used for validation studies to confirm the differential protein expression observed in the global discovery study. Since the majority of these proteins were in the day-6 treatment group, we used an independent set of day-6 samples for validation studies. In MRM, for each selected protein, one unique peptide and one pair of precursor and product ions (MRM transitions) were selected. Relative quantity and specificity of these MRM peptides/transitions are illustrated in **Fig. 3-1 and 3-2**. Synthetic standard peptides were first tested to confirm the transition ion pairs derived from Skyline. Results showed that among the 4 proteins changed in the day-6 treatment group, prostate-associated microseminoprotein (MSMP) was found significantly decreased when the FO treatment group was compared to control (63.3% lower expression in the FO group, p=0.0242). Down-regulation of MSMP in the OA treated group was not significant (28.4% lower expression, p= 0.3474), (**Fig. 3-7 A**). Since MSMP is also a secreted protein, we tested expression in CM of PC3 cell grown in the presence of FO, OA or vehicle. Western blot results show that secreted MSMP in

CM appears as a single band around 16kD. FO treated PC3 cells secreted less MSMP compared to control (p=0.0096) or OA group (p=0.029) (**Fig. 3-8 F** & **G**).

The other protein, sterol carrier protein 2 (SCP2), was also found to be significantly up-regulated in the FO treated group compared to control (47.1% over-expressed in FO group, p=0.001), or OA (27.8%FO, p= 0.0113) (**Fig. 3-7 B**). However, unlike the global proteome results, there was no significant change between OA and C. No significant changes were observed in spiked-in chicken lysozyme and RPL6, which served as QA/QC samples (constant amount among three groups observed) (**Fig. 3-7 C & D**).

SQSTM1, a protein involved in autophagy, was found to be over-expressed in FO group one day after the treatment in our global proteomic study. Western blot results were consistent with the LC/MS results that demonstrated SQSTM1 levels in the FO group were 2.1-fold higher than that in the control group (p=0.042) (**Fig. 3-8 A & B**). Although the proteomic analysis also showed a higher level of expression in FO group when FO and OA groups were compared (fold-change=2.35), the change, however, was not statistically significant (p=0.0867) based on our p=0.05 cutoff. There was no significant difference between OA and control. Real-time PCR results showed that at the transcriptional level, mRNA expression of SQSTM1 in FO treated group was higher than both control (fold-change=2.16, p=0.003) and OA treated group (fold-change=2.26, p=0.003) (**Fig. 3-8 C**). To further investigate the function of autophagy, autophagy inhibitor chloroquine (CQ) was applied to cells with different FA treatment, and LC3 I/II levels were measured by Western blot. The results indicated that, under the condition of autophagy suppression induced by CQ, LC3 II levels accumulated significantly (two-way

ANOVA, p=0.0005) while LC3 I levels were not affected. However, no significant LC3 II changes were observed among FA treatment groups (two-way ANOVA, p=0.7853) (**Fig. 3-8 D & E**), suggesting that autophagy may not be significantly inhibited under this experimental condition.

5) MSMP expression through cell lines

MSMP expression level was examined in both cell lysates and conditioned media (CM) from other prostate cancer cell lines LNCaP, PPC1, LAPC4, C4-2, DU145 and human embryonic kidney cell line HEK293, and also cell lysates from normal prostate epithelial cell line RWPE1. PC3 cell line was used as positive control (**Fig. 3-9**). Our results show that among these cell lines, strong MSMP expression can only be detected in PC3 and PPC1, which is considered to be a sub-cell line of PC3. Also a molecular weight difference can be observed that MSMP in cell lysate is smaller compared to its secreted counterpart. The slight difference might be caused by the signal peptides direct secreted MSMP to be transported outside the cell membrane.

6) Proteasomal proteolysis activity under different FA treatment

Our study has shown that FO could upregulate SQSTM1, which is required by autophagy. Although the upregulation starts from the transcriptional level, we are still curious if the degradation process is also affected by FO or not. The DQ-BSA assay was then performed to test whether FO can regulate proteasomal proteolysis activity. When cells intake and digest DQ-BSA into smaller pieces, the self-quench effect of DQ-BSA is stopped and under excitation light (the same wavelength to detect Rhodamine Red dye), red dots in cells are evident. **Fig. 3-10** shows that most cells in all treatments have intracellular red dots, suggesting FO may not change the proteasomal proteolysis activity.

4 Discussion

In this study, we investigated the different molecular pathways of OA and FO action in PCa cells. Previous investigations of n-3 PUFAs and PCa have pointed to some of the potential mechanisms of FO on PCa growth inhibition. One study detected down-regulated AR protein expression in LNCaP cells after 10 weeks continuous 20 μM DHA treatment [123], while another study did not detect a AR protein change in LNCaP cells treated by DHA at concentrations as high as 180 μM for 24 to 36 hrs [191], suggesting differential regulatory changes over time may have occurred during the treatment periods. One point worth noting is that the concentration of the fatty acids used for this study is based on our preliminary experiments and other reported studies [192-194]. At 100 μM, FASN activity is significantly reduced by OA while cell viability remains unaffected. However, *in vivo* human exposure to this concentration of FAs may never reach 100 μM due to its poor water solubility and lipid uptake limitations. Thus the actual exposure of the cells to FAs may be much lower than the calculated 100 μM concentration.

Our results suggest that only FO, not OA, suppresses PC3 cell viability, and this inhibition occurred as early as one day after the treatment. Hierarchy analysis of global proteomic data generated a group of proteins in which the majority of proteins were increased after 24-hr FO treatment. Specifically, both SQSTM1, and shock 70 kDa proteins A1A/A1B are connected to autophagy [189, 190] and both were significantly elevated one day after FO treatment but with no change in the OA treated group. This result indicates FO, not OA, triggers rapid stress-responding reactions, which may explain the early event of inhibition of viability. The elevated expression of SQSTM1, an autophagy associated protein, was also validated by Western Blot (**Fig. 3-8A & B**).

Autophagy activation is critical for rapid proliferating tumor cells in response to cellular stress and high metabolic demands. In the dynamic process of autophagy, SQSTM1 is required for the degradation of polyubiquitinated substrates, and SQSTM1 itself is also degraded during this process. The accumulating SQSTM1 level implicates a possible inhibition of autophagy, which in turn causes the accumulation of SQSTM1 that should be otherwise degraded in a normal autophagy process, suppressing the growth of the tumor cells. Consistent with our result, one study also found elevated SQSTM1 12-hr after the treatment of EPA in promyelocytic cells [195]. Conversely, Shin *et al*. showed that autophagy, in this case acting as a tumor suppressor, was activated by DHA and played a critical role in apoptosis of PC3 cells induced by DHA [196]. However, in investigating the cause of SQSTM1 elevation under FO treatment, we found this increased protein expression was most likely regulated by transcriptional mechanism rather than reduced post-translational degradation, implicating other transcription regulator(s) may be involved. It has been previously reported that oxidative stress can induce SQSTM1 at the transcriptional level, and SQSTM1 triggers antioxidant effects [49]. Since FO was found to induce oxidation stress and cytotoxicity [50], we suggest that the observed increase of SQSTM1 might take place at the stage when cells are starting to respond to oxidative stress.

Our study suggests that although OA suppressed FA synthesis rate to near 50% on day 6, FASN protein level showed no significant change in the OA group compared to control. One study in C6 glioma cells suggested OA was a stronger inhibitor than n-3 PUFA and other FAs in FA synthesis [197]. Consistent with their results and another study in 1999 [198], we found no protein level changes in FASN expression.

Interestingly, they discovered that OA down-regulated the expression of another key enzyme in FA synthesis, acetyl-coenzyme A carboxylase, which is involved in the formation of malonyl-CoA. In contrast, FASN expression levels in FO treated group are decreased compared to OA group (t-test, p=0.05, multiple comparison test p=0.1), indicating additional transcriptional level modulation of FASN was triggered by FO.

Our quantitative global proteomic study also revealed that a group of glycolysis/gluconeogenesis enzymes were changed in FO treated cells and four of the enzymes were significantly down-regulated in the 6-day treatment group. Although most of the decreased enzymes are shared by both glycolysis and gluconeogenesis, PKM specifically catalyzes a rate-limiting step in glycolysis. In PCa, glucose is not a major bioenergy source as in many other cancer types [93], therefore rendering FDG-PET imaging ineffective for PCa tumor imaging [199]. However, a study based on NIH dbEST database showed PCa tissue has 9 out of 10 glycolysis genes overexpressed compared to normal tissue [54]. Additionally, some glycolytic enzymes, PKM and PGAM1, are expressed differently between PCa and normal tissue by isoform or post-translational modification (PTM) level [200]. PCa cell sensitivity to stress is enhanced when glycolysis is suppressed [201]. This suggests glycolysis still plays a role in PCa even though the glucose uptake is low. Expression of hepatic pyruvate kinase [202] and glucose-6-phosphate dehydrogenase [203] were also reportedly blocked by n-6 or n-3 PUFAs. One of the possible mechanisms is through prevention of chREBP translocating to nuclei [204]. In the context of cancer cells, the inhibition may be mediated by liver kinase B1 (LKB) [205]. Effects of PUFAs on glycolysis are tissue specific so that in skeletal muscle cells, the process is reversed [193]. Our study indicates FO may suppress

glycolysis by inhibition of expression of multiple enzymes, suggesting that in the PC3 cell line, decreases in *de novo* FA synthesis in later time points may be due to lower levels of pyruvate supplied by glycolysis. To the contrary, prolonged OA treatment does not suppress glycolytic enzyme expression and we therefore conclude that FO and OA may inhibit FA synthesis through different pathways, which are dependent or independent of glycolysis down-regulation, respectively.

In addition to involvement of glycolytic enzymes, additional potential targets of FO were investigated and validated in independent sets of samples. Among the five candidate proteins in the 6-day treatment arm, the increase of SCP2 and decrease of MSMP was validated by MRM. In the global study, SCP2 was elevated in both FO and OA, with the FO group demonstrating significantly higher expression than OA and control samples. This result was confirmed by Western Blot analysis, although the SCP2 increase in OA group was not found to be significant. Functionally it is expected that this lipid carrier protein expression would increase when exogenous FAs are added to the medium. The other protein, consistent in validation experiments, MSMP, is a highly conserved protein and belongs to beta-microseminoprotein family and its expression was observed in both benign and tumor tissues. The immunohistochemical analysis also demonstrated that tumor MSMP expression is different from the pattern in benign tissue [206]. As a recently identified protein, MSMP has not been well studied. One report suggested MSMP has a role in inflammation as a ligand binding to CC chemokine receptor 2B (CCR2B) and acts as a chemoattractant. MSMP binding therefore induces the migration of peripheral blood monocytes (PBM) and peripheral blood lymphocyte (PBL) cells but not polymorphonuclear neutrophils (PMN) cells [207], indicating a pro-

inflammation role of MSMP. MSMP was also found to stimulate p-ERK, which plays a central signaling role in cancer progression [207, 208]. Our results demonstrate that FO can lower MSMP levels, offering an additional explanation for the anti-inflammation action of n-3 FAs in prostate cancer. Taken together, FO and OA both suppress FA synthesis activity but only FO inhibits cell survival. The global proteomic studies and follow-up validation demonstrated that FO modulates protein levels of SQSTM1, MSMP, and SCP2 in PCa cells, suggesting a possible longitudinal regulation by FO: inducing cytotoxicity after short treatment; suppressing inflammation pathways and inhibiting the glycolytic pathway with prolonged treatment.

Table 3-1 Chicken lysozyme is used as an external quality control/quality assurance protein in label-free quantifications.

CV (%) of the double injection of each sample, as well as CV (%) of 5 samples X 2 injections in each treatment groups is shown.

CV (%) between injections in individual sample									
CN12A	CN12B	CN13A	CN13B	CN14A	CN14B	CN15A	CN15B	CN16A	CN16B
8.51282325		11.09747533		0.647072068		2.995175413		2.934507782	
CN62A	CN62B	CN63A	CN63B	CN64A	CN64B	CN65A	CN65B	CN66A	CN66B
3.785901709		23.73699549		0.641360163		0.218983012		1.565582643	
TA11A	TA11B	TA13A	TA13B	TA14A	TA14B	TA15A	TA15B	TA16A	TA16B
8.216934671		6.499618397		0.840747928		3.146318445		4.509634082	
TA62A	TA62B	TA63A	TA63B	TA64A	TA64B	TA65A	TA65B	TA66A	TA66B
3.660808264		7.033933843		2.608942409		0.8545686		0.382488038	
TF12A	TF12B	TF13A	TF13B	TF14A	TF14B	TF15A	TF15B	TF16A	TF16B
14.77135129		10.40685476		0.888087107		0.141211279		0.039087138	
TF61A	TF61B	TF62A	TF62B	TF64A	TF64B	TF65A	TF65B	TF66A	TF66B
12.99787707		3.926032974		5.949187586		2.595616316		2.62955399	

CV (%) among injections in every sample

CV(%)_CN1	CV(%)_CN6	CV(%)_TA1	CV(%)_TA6	CV(%)_TF1	CV(%)_TF6
9.5	11	12.7	4.9	8.5	4.9

Table 3-2 Label-free quantification information for the 127 significantly changed proteins.

Label-free quantification information for the 127 significantly changed proteins.

CN=control; TF=FO treated; TA=OA treated; FC=Fold change.

Protein ID	Gene Name	Protein Name	FC_TA1_CN1	FC_TF1_CN1	FC_TF1_TA1	FC_TA6_CN6	FC_TF6_CN6	FC_TF6_TA6	ANOVA p-value	q-value
Q1L6U9	MSMP	Prostate-associated microseminoprotein	-1.1	1	1.1	-1	-2	-2	1.80E-08	2.12E-05
Q00796	SORD	Sorbitol dehydrogenase	-1.1	1.1	1.1	1.1	1.5	1.4	1.97E-07	1.16E-04
Q96EP5-2	DAZAP	Isoform 2 of DAZ-associated protein 1	1	1	-1	-1	-1	-1	4.40E-07	1.73E-04
E7EMC7	SQSTM1	Sequestosome-1	-1	1.7	1.8	1.1	1.2	1	1.10E-06	3.23E-04
K7ELW0	PARK7	Protein DJ-1	-1	-1	-1	1.1	-1.1	-1.2	2.41E-06	5.27E-04
P62937	PPIA	Peptidyl-prolyl cis-trans isomerase A	-1	-1	-1	1.1	-1.2	-1.2	2.98E-06	5.27E-04
B4DQR8	ZYX	Zyxin	-1.1	-1.1	-1	1.2	-1.3	-1.5	3.13E-06	5.27E-04
Q13501-2	SQSTM	Isoform 2 of Sequestosome-1	-1	1.6	1.6	1.1	1.1	1	4.12E-06	6.06E-04
Q99497	PARK7	Protein DJ-1	-1	-1	-1	1	-1.2	-1.2	5.78E-06	7.10E-04
J3QSB5	RPL36	60S ribosomal protein L36	-1	-1	-1	1.1	-1.3	-1.5	6.03E-06	7.10E-04
P14866-2	HNRNP	Isoform 2 of Heterogeneous nuclear ribonucleoprotein L	-1	-1	-1	1.1	-1.2	-1.4	1.47E-05	1.57E-03
Q6IBS0	TWF2	Twinfilin-2	-1	-1.1	-1.1	1.1	-1.3	-1.4	2.26E-05	2.19E-03
B4E0P1	PSMB7	Proteasome (Prosome, macropain) subunit, beta type, 7, isoform CRA_b	-1.2	-1.1	1.1	1	-1.3	-1.3	2.46E-05	2.19E-03

Q96AB3-2	ISOC	Isoform 2 of Isochorismatase domain-containing protein 2, mitochondrial	-1.1	-1.2	-1.1	1.1	-1.3	-1.4	2.60E-05	2.19E-03
D6RG15	TWF2	Twinfilin-2	-1	-1.1	-1.1	1.1	-1.4	-1.5	4.15E-05	3.26E-03
E9PEP6	NRP1	Neuropilin-1	-1	1	1.1	1.2	-1.2	-1.4	5.48E-05	3.43E-03
Q13509	TUBB3	Tubulin beta-3 chain	1	-1	-1.1	1.1	-1.2	-1.3	5.60E-05	3.43E-03
Q99436	PSMB7	Proteasome subunit beta type-7	-1.1	-1.1	1	1	-1.2	-1.3	5.84E-05	3.43E-03
G3V1X9	CBX5	Chromobox homolog 5 (HP1 alpha homolog, Drosophila), isoform CRA_a	1	-1.1	-1.1	-1.1	-1.3	-1.2	6.06E-05	3.43E-03
P14174	MIF	Macrophage migration inhibitory factor	-1	-1.1	-1.1	1.1	-1.5	-1.6	6.15E-05	3.43E-03
H3BT58	COTL1	Coactosin-like protein	1	1.1	1	1.1	1.2	1.1	6.32E-05	3.43E-03
P33993-3	MCM	Isoform 3 of DNA replication licensing factor MCM7	1	1	-1	-1	-1	-1	6.94E-05	3.43E-03
P06744	GPI	Glucose-6-phosphate isomerase	1	1	1	1.1	-1.2	-1.2	7.22E-05	3.43E-03
Q15067-3	ACOX	Isoform 3 of Peroxisomal acyl-coenzyme A oxidase 1	-1.2	1.2	1.5	1.1	1.5	1.3	7.29E-05	3.43E-03
P14618	PKM	Pyruvate kinase isozymes M1/M2	-1	-1	1	1	-1.2	-1.2	7.44E-05	3.43E-03
A6NNI4	CD9	CD9 antigen	-1.1	1.3	1.4	1.1	1.8	1.6	7.58E-05	3.43E-03
Q15067-2	ACOX	Isoform 2 of Peroxisomal acyl-coenzyme A oxidase 1	-1.2	1.2	1.5	1.1	1.4	1.3	8.71E-05	3.80E-03
B4DGP8	CANX	Calnexin	-1.1	-1	1	1.1	-1.3	-1.4	9.08E-05	3.82E-03
P31153	MAT2A	S-adenosylmethionine synthase	-1.1	-1	1.1	1.1	1.3	1.1	1.00E-04	4.07E-03

		isoform type-2								
P22307-4	SCP	Isoform 4 of Non-specific lipid-transfer protein	-1.1	-1.1	-1	1.3	1.7	1.3	1.08E-04	4.22E-03
P08195-2	SLC3A	Isoform 2 of 4F2 cell-surface antigen heavy chain	-1	1.5	1.5	-1.1	-1.2	-1.1	1.20E-04	4.44E-03
P14866	HNRNPL	Heterogeneous nuclear ribonucleoprotein L	-1	-1	1	1.1	-1.2	-1.3	1.21E-04	4.44E-03
A6NJG9	PSME1	Proteasome activator complex subunit 1	-1	-1.1	-1.1	1	-1.6	-1.6	1.26E-04	4.51E-03
B3KS98	EIF3S3	Eukaryotic translation initiation factor 3 subunit H	1.1	-1.1	-1.2	1.1	-1.2	-1.3	1.35E-04	4.67E-03
Q9BR76	CORO1B	Coronin-1B	1	1	-1	1.1	-1.3	-1.4	1.51E-04	5.07E-03
O14786-2	NRP	Isoform 2 of Neuropilin-1	-1.1	1	1.1	1.1	-1.5	-1.6	1.76E-04	5.74E-03
Q9Y3U8	RPL36	60S ribosomal protein L36	-1	-1.1	-1.1	1	-1.3	-1.3	1.84E-04	5.86E-03
E7EUT5	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	-1	-1	1	1.1	-1.3	-1.4	2.03E-04	6.23E-03
P00558	PGK1	Phosphoglycerate kinase 1	-1.1	1	1.1	1.1	-1.3	-1.4	2.06E-04	6.23E-03
P68371	TUBB4B	Tubulin beta-4B chain	1	-1	-1	1.1	-1.1	-1.2	2.18E-04	6.42E-03
P11940-2	PABPC	Isoform 2 of Polyadenylate-binding protein 1	-1	-1	-1	1.1	-1.2	-1.3	2.36E-04	6.72E-03
Q15942	ZYX	Zyxin	-1	-1.1	-1.1	1.2	-1.2	-1.4	2.40E-04	6.72E-03
Q12906-2	ILF	Isoform 2 of Interleukin enhancer-binding factor 3	-1	-1	1	1.1	-1.2	-1.3	3.02E-04	8.28E-03
Q15067	ACOX1	Peroxisomal acyl-coenzyme A oxidase 1	-1.1	1	1.1	1.1	-1.1	-1.2	3.14E-04	8.41E-03
Q16658	FSCN1	Fascin	-1	-1	-1	1.1	-1.2	-1.4	3.53E-04	9.24E-03
E7EUT4	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	-1	1	1	1.1	-1.2	-1.4	3.88E-04	9.92E-03

P09429	HMGB1	High mobility group protein B1	-1	1	1	1.2	-1.1	-1.3	4.31E-04	1.08E-02
E5RID6	DECR1	2,4-dienoyl-CoA reductase, mitochondrial	-1	-1.2	-1.2	1.2	-1.2	-1.4	4.62E-04	1.13E-02
P04406	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	-1	-1	1	1.1	-1.2	-1.3	4.93E-04	1.18E-02
O00571-2	DDX3	Isoform 2 of ATP-dependent RNA helicase DDX3X	-1.1	-1.1	-1	1	-1.3	-1.3	5.31E-04	1.23E-02
B7Z6B8	DECR1	2,4-dienoyl-CoA reductase, mitochondrial	-1	-1.1	-1.1	1.2	-1.2	-1.4	5.36E-04	1.23E-02
Q5T7C6	HMGB1	High mobility group protein B1 (Fragment)	-1	1	1	1.2	-1.1	-1.3	5.48E-04	1.23E-02
P00441	SOD1	Superoxide dismutase [Cu-Zn]	-1	1	1	1.1	-1.1	-1.2	5.54E-04	1.23E-02
B4DNL6	PRPS1	Ribose-phosphate pyrophosphokinase	1	-1	-1.1	1.1	-1.2	-1.3	5.84E-04	1.26E-02
O15372	EIF3H	Eukaryotic translation initiation factor 3 subunit H	1.1	-1.1	-1.2	1.1	-1.2	-1.3	5.94E-04	1.26E-02
H0Y5E8	SAR1A	GTP-binding protein SAR1a (Fragment)	1.1	1.1	-1	1.2	-1.2	-1.5	5.98E-04	1.26E-02
B4DQ19	SAR1A	GTP-binding protein SAR1a	1	1.1	1	1.2	-1.2	-1.5	6.60E-04	1.34E-02
P52292	KPNA2	Importin subunit alpha-2	1	1.1	1	1.1	1.2	1.1	6.70E-04	1.34E-02
K7ELJ7	CAPNS1	Calpain small subunit 1	1.1	-1.1	-1.1	1.1	-1.2	-1.2	6.80E-04	1.34E-02
P40121-2	CAP	Isoform 2 of Macrophage-capping protein	-1	-1.1	-1.1	1.1	-1.1	-1.2	6.86E-04	1.34E-02
A7MAP0	CORO1C	Coronin	-1	-1	1	1.1	-1.2	-1.3	6.94E-04	1.34E-02
P68363	TUBA1B	Tubulin alpha-1B chain	1	1	-1	1.1	-1.2	-1.4	7.18E-04	1.36E-02
P30086	PEBP1	Phosphatidylethanolamine-binding	-1	1	1.1	1	-1.1	-1.1	8.05E-04	1.49E-02

		protein 1								
P06733	ENO1	Alpha-enolase	-1	-1.1	-1	1	-1.2	-1.2	8.15E-04	1.49E-02
B1ALA9	PRPS1	Ribose-phosphate pyrophosphokinase 1	1	-1	-1.1	1.1	-1.2	-1.3	8.25E-04	1.49E-02
P04899-4	GNAI	Isoform sGi2 of Guanine nucleotide-binding protein G(i) subunit alpha-2	1	1	-1	1.2	-1.2	-1.4	8.50E-04	1.52E-02
Q13630	TSTA3	GDP-L-fucose synthase	1.1	1.4	1.3	1.1	-1.3	-1.4	1.05E-03	1.85E-02
P09104	ENO2	Gamma-enolase	-1	1.1	1.2	1.1	1	-1.1	1.11E-03	1.93E-02
J3KQ32	OLA1	Obg-like ATPase 1	-1.2	1	1.2	1	-1.5	-1.5	1.13E-03	1.94E-02
H7C463	IMMT	Mitochondrial inner membrane protein (Fragment)	1.1	1.1	-1	1.1	-1.2	-1.3	1.19E-03	2.00E-02
P07737	PFN1	Profilin-1	-1.1	-1	1	1.1	-1.1	-1.2	1.24E-03	2.06E-02
P33316-2	DU	Isoform 2 of Deoxyuridine 5'-triphosphate nucleotidohydrolase, mitochondrial	-1	1.1	1.1	1.1	-1.3	-1.5	1.63E-03	2.66E-02
C9J406	IMMT	Mitochondrial inner membrane protein	1.1	1.1	-1	1.1	-1.2	-1.3	1.65E-03	2.66E-02
Q92597-2	NDRG	Isoform 2 of Protein NDRG1	-1	1.1	1.1	1.3	-1.2	-1.5	1.86E-03	2.93E-02
P22061-2	PCMT	Isoform 2 of Protein-L-isoaspartate(D-aspartate) O-methyltransferase	1.1	1	-1	1	-1.1	-1.1	1.87E-03	2.93E-02
P14618-2	PK	Isoform M1 of Pyruvate kinase isozymes M1/M2	-1	-1	1	1.1	-1.2	-1.3	1.93E-03	2.99E-02
F5GX11	PSMA1	Proteasome subunit alpha type-1	1.1	1.2	1.1	-1	-1.2	-1.1	2.02E-03	3.05E-02
P55060-	CSE1	Isoform 3 of	-1.1	-1.1	-1	-1	-1.3	-1.2	2.05E	3.05

3		Exportin-2							-03	E-02
Q16891-3	IMM	Isoform 3 of Mitochondrial inner membrane protein	1.1	1.1	-1	1.1	-1.2	-1.3	2.05E-03	3.05E-02
C9J4N6	IDH1	Isocitrate dehydrogenase [NADP] cytoplasmic (Fragment)	1	1	1	-1	-1.3	-1.2	2.20E-03	3.20E-02
G3V5P4	CFL2	Cofilin 2 (Muscle), isoform CRA_d	-1	1	1	1.1	-1.2	-1.3	2.22E-03	3.20E-02
J3QL06	HYOU1	Hypoxia up-regulated protein 1 (Fragment)	1	-1.4	-1.4	1	1.2	1.1	2.23E-03	3.20E-02
H0YKK6	PSME1	Proteasome activator complex subunit 1	1	-1	-1	-1.1	-1.4	-1.3	2.32E-03	3.29E-02
O60271-3	SPAG	Isoform 3 of C-Jun-amino-terminal kinase-interacting protein 4	-1.1	1	1.1	1.3	-1.1	-1.4	2.42E-03	3.31E-02
F8VSA6	NEDD8	NEDD8	-1	-1	-1	1.1	-1.1	-1.1	2.42E-03	3.31E-02
B4DDJ7	RAD23A	UV excision repair protein RAD23 homolog A	1.1	1	-1	1.1	-1.4	-1.5	2.43E-03	3.31E-02
F8VRG9	PCBP2	Poly(rC)-binding protein 2	1	-1	-1	1.2	-1	-1.2	2.45E-03	3.31E-02
Q04637-3	EIF4G	Isoform B of Eukaryotic translation initiation factor 4 gamma 1	-1	-1	-1	1.1	-1.1	-1.2	2.48E-03	3.32E-02
Q01105-2	SE	Isoform 2 of Protein SET	1	-1	-1	1.1	-1.2	-1.3	2.56E-03	3.33E-02
Q16891-2	IMM	Isoform 2 of Mitochondrial inner membrane protein	1.1	1.1	-1	1.1	-1.2	-1.3	2.61E-03	3.33E-02
P35754	GLRX	Glutaredoxin-1	1	1	1	1.1	-1.3	-1.4	2.63E-03	3.33E-02
B7Z1R5	ATP6V1A	V-type proton ATPase	-1	1.1	1.2	1.2	-1	-1.2	2.63E-03	3.33E-02

		catalytic subunit A								
F8W904	CSE1L	Exportin-2	-1.1	-1.1	1	-1	-1.3	-1.2	2.64E-03	3.33 E-02
P60891	PRPS1	Ribose-phosphate pyrophosphokinase 1	-1.1	-1	1.1	1.1	-1.2	-1.3	2.66E-03	3.33 E-02
C9IZ80	BZW1	Basic leucine zipper and W2 domain-containing protein 1 (Fragment)	-1	1.1	1.1	1.2	-1.1	-1.3	2.74E-03	3.37 E-02
Q8IVF2-3	AHNAK	Isoform 3 of Protein AHNAK2	1	-1.1	-1.1	1.1	-1.1	-1.3	2.76E-03	3.37 E-02
P17655	CAPN2	Calpain-2 catalytic subunit	1.1	1	-1.1	1.1	-1.1	-1.2	2.78E-03	3.37 E-02
G3V1A4	CFL1	Cofilin 1 (Non-muscle), isoform CRA_a	-1.1	-1	1	1.1	-1.1	-1.3	2.84E-03	3.41 E-02
P13640-2	MT1	Isoform 2 of Metallothionein-1G	1.1	-1.2	-1.3	1.2	1.2	1	2.89E-03	3.44 E-02
Q9BUF5	TUBB6	Tubulin beta-6 chain	1	1.1	1.1	1.2	1	-1.1	2.99E-03	3.52 E-02
C9JIF9	APEH	Acylamino-acid-releasing enzyme	-1	1.1	1.1	1.1	-1.2	-1.3	3.07E-03	3.58 E-02
P08107	HSPA1A	Heat shock 70 kDa protein 1A/1B	-1	1.4	1.5	1	-1.2	-1.2	3.11E-03	3.59 E-02
P23526	AHCY	Adenosylhomocysteinase	-1	-1	-1	1.1	-1.1	-1.2	3.19E-03	3.65 E-02
P05388	RPLP0	60S acidic ribosomal protein P0	-1	1	1.1	1.2	-1.1	-1.3	3.34E-03	3.72 E-02
P02452	COL1A1	Collagen alpha-1(I) chain	-1	-1	-1	1.1	-1.2	-1.3	3.35E-03	3.72 E-02
B4DVE7	ANXA11	Annexin	-1	1.1	1.1	1.2	1.1	-1.1	3.35E-03	3.72 E-02
P62942	FKBP1A	Peptidyl-prolyl cis-trans isomerase FKBP1A	-1.1	1	1.1	1.1	-1.1	-1.2	3.38E-03	3.72 E-02
P37837	TALDO1	Transaldolase	-1	1	1	1.1	-1.1	-1.2	3.56E-03	3.87 E-02
B4DUW4	SUMO3	Small ubiquitin-related modifier 3	1	-1.1	-1.2	1.3	1	-1.2	3.80E-03	4.09 E-02

P49411	TUFM	Elongation factor Tu, mitochondrial	1	1.1	1	1.3	-1.3	-1.7	3.83E-03	4.09E-02
O75396	SEC22B	Vesicle-trafficking protein SEC22b	-1	1.1	1.1	1.2	-1	-1.3	3.86E-03	4.09E-02
P62140	PPP1CB	Serine/threonine-protein phosphatase PP1-beta catalytic subunit	-1.1	-1.1	-1	-1	-1.2	-1.2	4.01E-03	4.20E-02
Q13642-3	FHL	Isoform 3 of Four and a half LIM domains protein 1	-1	1.1	1.1	1.2	1.2	-1	4.04E-03	4.20E-02
P22234	PAICS	Multifunctional protein ADE2	1	1	1	1.1	-1.1	-1.2	4.10E-03	4.20E-02
Q8WU40	CAMK2G	CAMK2G protein (Fragment)	-1	1	1.1	1.1	-1.2	-1.3	4.11E-03	4.20E-02
Q04760	GLO1	Lactoylglutathione lyase	-1.1	-1.1	-1	1	-1.3	-1.3	4.19E-03	4.25E-02
Q9BRA2	TXNDC17	Thioredoxin domain-containing protein 17	1.1	1	-1.1	1.1	-1.2	-1.3	4.69E-03	4.67E-02
E9PPQ8	NASP	Nuclear autoantigenic sperm protein (Fragment)	1.1	-1.1	-1.1	1.1	1.2	1.1	4.70E-03	4.67E-02
Q9NYU2-2	UGGT	Isoform 2 of UDP-glucose:glycoprotein glucosyltransferase 1	1.1	1.1	-1	-1	-1.5	-1.4	4.73E-03	4.67E-02
C9JE81	HADHB	3-ketoacyl-CoA thiolase (Fragment)	-1	-1.1	-1	1.1	-1.2	-1.4	4.76E-03	4.67E-02
O15231-3	ZNF18	Isoform 3 of Zinc finger protein 185	-1	-1	-1	1	-1.3	-1.3	4.85E-03	4.72E-02
F5GWY2	ATIC	Phosphoribosylaminoimidazolecarboxamide formyltransferase	-1	1	1.1	1.1	-1.1	-1.2	4.92E-03	4.73E-02
P04075	ALDOA	Fructose-bisphosphate aldolase A	-1.1	1	1.1	1.1	-1.1	-1.2	4.94E-03	4.73E-02
Q9NUQ9	FAM49B	Protein FAM49B	-1	-1.1	-1.1	1	-1.3	-1.3	5.22E-03	4.92E-02

Q9BXP5-2	SRR	Isoform 2 of Serrate RNA effector molecule homolog	1	1.2	1.2	1.2	-1	-1.2	5.24E-03	4.92E-02
D6RD66	WDR1	WD repeat-containing protein 1 (Fragment)	-1	-1	-1	1.2	-1.1	-1.3	5.27E-03	4.92E-02
C9JLK2	APEH	Acylamino-acid-releasing enzyme (Fragment)	-1	1.1	1.1	-1.2	-1.6	-1.3	5.33E-03	4.94E-02

Table 3-3 Differentially expressed proteins in cluster A and B.

(*: t-test p≤0.05, **: t-test p≤0.01, n=5)

Genes	Protein name	Log2 FO/CN (day1)	Log2 OA/CN (day1)	Log2 FO/CN (day6)	Log2 OA/CN (day6)	ANOVA p-value	q-value
Cluster A							
SCP2	Non-specific lipid-transfer protein	-0.12	-0.10	0.75**	0.36**	1.08E-04	4.22E-03
CD9	CD9 antigen	0.39	-0.10	0.81**	0.12	7.58E-05	3.43E-03
ACOX	Isoform 2 of Peroxisomal acyl-coenzyme A oxidase 1	0.25	-0.29	0.52**	0.14	8.71E-05	3.80E-03
ACOX1	Acyl-coenzyme A oxidase	0.30	-0.28	0.55**	0.12	7.29E-05	3.43E-03
SORD	Sorbitol dehydrogenase	0.11	-0.09	0.59**	0.09	1.97E-07	1.16E-04
COTL1	Coactosin-like protein	0.11	0.05	0.31*	0.19	6.32E-05	3.43E-03
KPNA2	Importin subunit alpha-2	0.08	0.02	0.29*	0.17	6.70E-04	1.34E-02
Cluster B							
APEH	Acylamino-acid-releasing enzyme (Fragment)	0.11	-0.07	-0.67*	-0.28	5.33E-03	4.94E-02
TSTA3	GDP-L-fucose synthase	0.50*	0.12	-0.33*	0.12	1.05E-03	1.85E-02
SLC3A	Isoform 2 of 4F2 cell-surface antigen heavy chain	0.59**	-0.02	-0.26	-0.18	1.20E-04	4.44E-03
SQSTM1	Sequestosome-1	0.76**	-0.06	0.24	0.18	1.10E-06	3.23E-04
HSPA1A	Heat shock 70 kDa protein 1A/1B	0.53**	-0.05	-0.21	0.01	3.11E-03	3.59E-02

Table 3-4 IPA pathway analysis of cluster C

Molecular and Cellular Functions		
Name	p-value	Number of Molecules
Cellular Growth and Proliferation	6.61E-03 – 2.26E-08	28
Cellular Movement	6.61E-03 – 1.45E-06	19
Cellular Development	6.61E-03 – 2.10E-06	23
Carbohydrate Metabolism	6.13E-03 – 4.16E-06	8
Cellular Death and Survival	8.17E-03 – 4.42E-05	22
Top Canonical Pathways		
Name	p-value	Overlap
Glycolysis I (GAPDH, GPI, PGK1, PKM)	1.86E-07	4/25 (16%)
Gluconeogenesis I (GAPDH, GPI, PGK1)	1.78E-05	3/25 (12%)
Remodeling of Epithelial Adherens Junctions (TUBB3, TUBB4B, ZYX)	3.65E-04	3/68(4.4%)
EIF2 Signaling (EIF3H, PABPC1, PPP1CB, RPL36)	5.49E-04	4/185 (2.2%)
Breast Cancer Regulation by Stathmin1 (TUBB3, TUBB4B, PPP1CB, GNAI2)	6.19E-04	4/191 (2.1%)

Table 3-5 Candidate targets of FO or OA treatment

Protein ID	Gene Name	Protein name	FC OA/CN	MP OA/CN	FC FO/CN	MP FO/CN	FC FO/OA	MP FO/OA	ANOVA q Value
Day 1									
E7EMC7	SQSTM1	Sequestosome-1	-1	0.7	1.7	0.0001	1.8	0.0001	0.0003
Day 6									
P22307	SCP2	Non-specific lipid-transfer protein	1.3	0.005	1.7	0.0002	1.3	0.003	0.004
Q16658	FSCN1	Fascin	1.1	0.08	-1.2	0.05	-1.4	6.00E-05	0.009
B4DGP8	CANX	Calnexin	1.1	0.3	-1.3	0.02	-1.4	0.001	0.004
Q1L6U9	MSMP	Prostate-associated microseminoprotein	-1	1	-2	0.0009	-2	0.0008	2.12E-05

FC: fold change
MP: p value of multiple comparison test

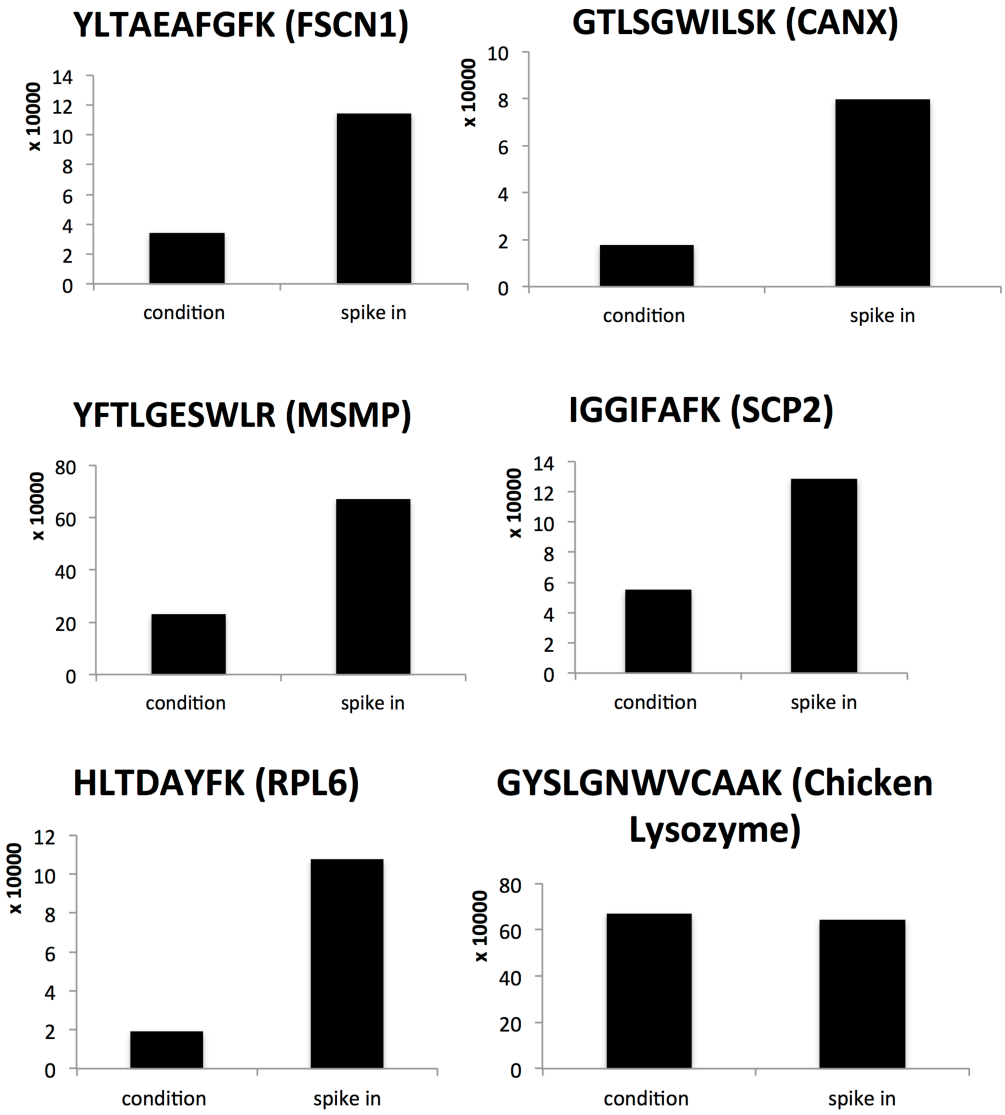
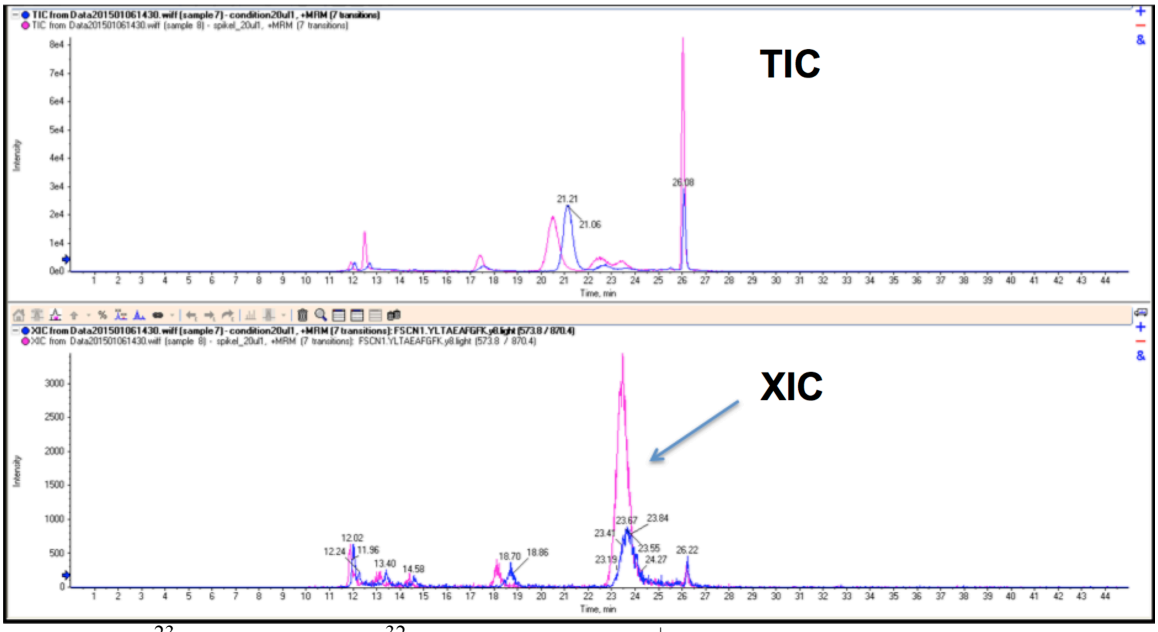


Figure 3-1 Synthetic peptides served as quantification standards for MRM assays.

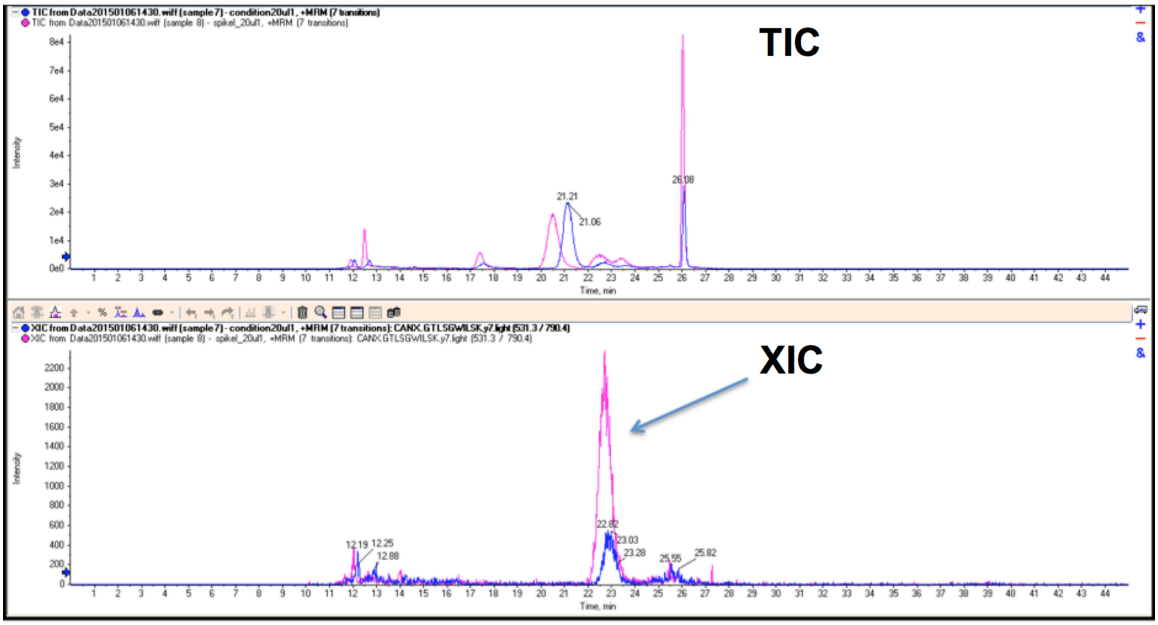
To confirm the specificity of the assay, these synthetic peptides were spiked into each cell lysate with known quantities and monitored by MRM transitions. The cell lysate without the spiked peptide is labeled as "condition", while the cell lysate with the spiked peptide is labeled as "spike in". Spiked-in ratio: every 90 µl PC3 cell lysate sample (11.25 µg total protein) was spiked in 0.2 ng ^{23}YLTAEAFGFK^{32} (FSCN1), 0.2 ng ^{78}GTLSGWILSK^{87} (CANX), 1.6 ng ^{52}YFTLGESWLR^{61} (MSMP), 0.1 ng ^{48}IGGIFAFK^{55} (SCP2) and 1.6 ng ^{211}HLTDAYFK^{218} (RPL6). For 20 µl protein was injected for each sample.



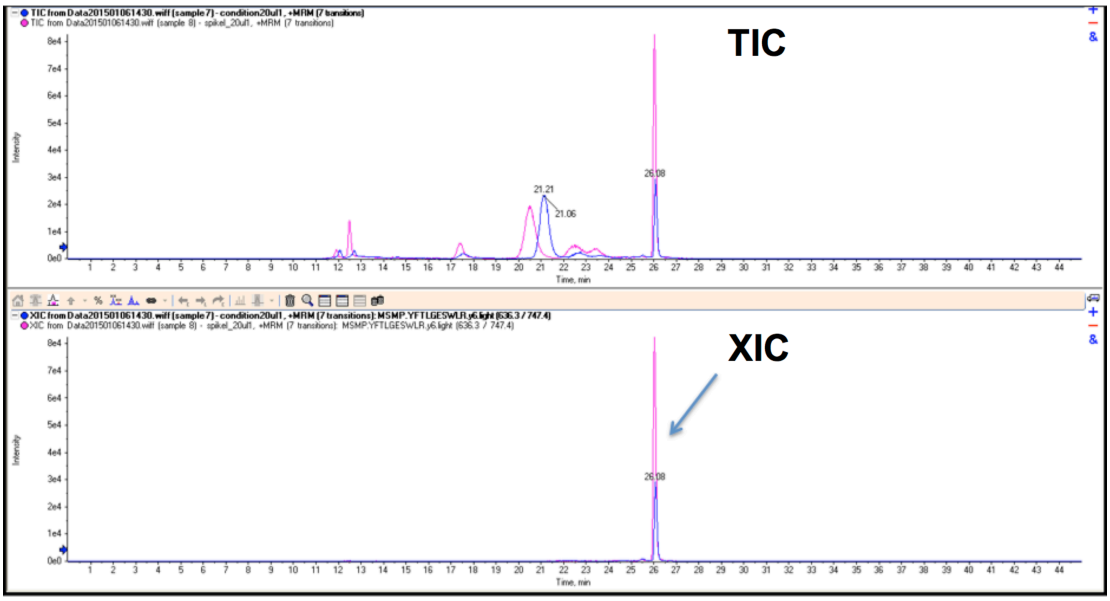
A. FSCN1, ^{23}YLTAEAFGFK^{32}, m/z 573.8 ($M+2H^+$) → m/z 870.4

Figure 3-2 XIC with (pink line) or without (blue line) spiked-in synthetic peptide in PC3 cell extracts.

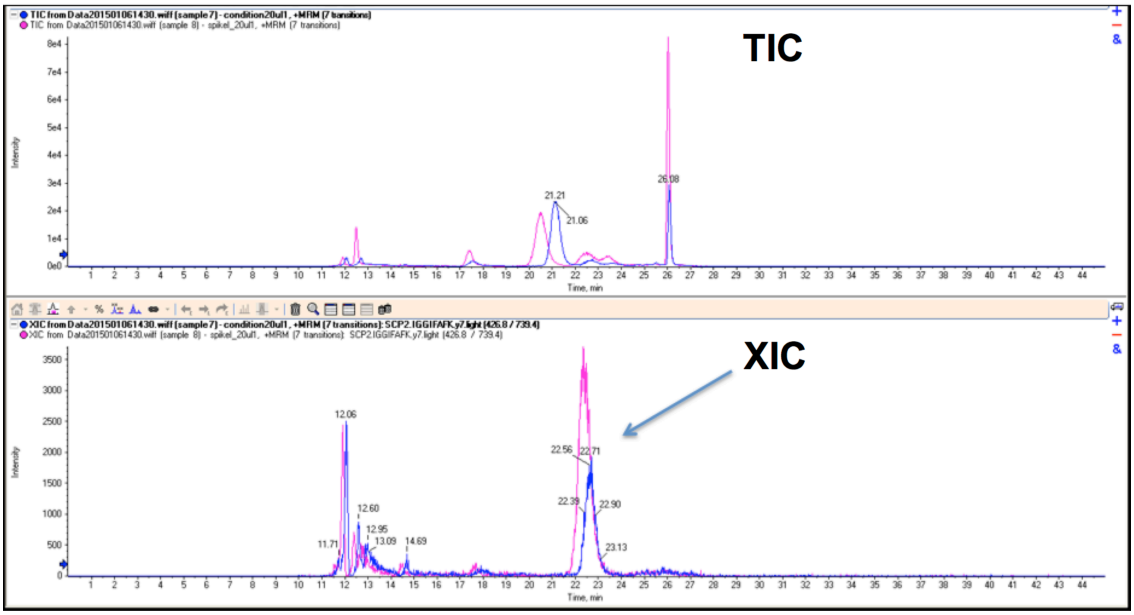
See Fig. 3-1 for relative quantity of each MRM peptide. (TIC: total ion chromatogram; XIC: extracted ion chromatogram)



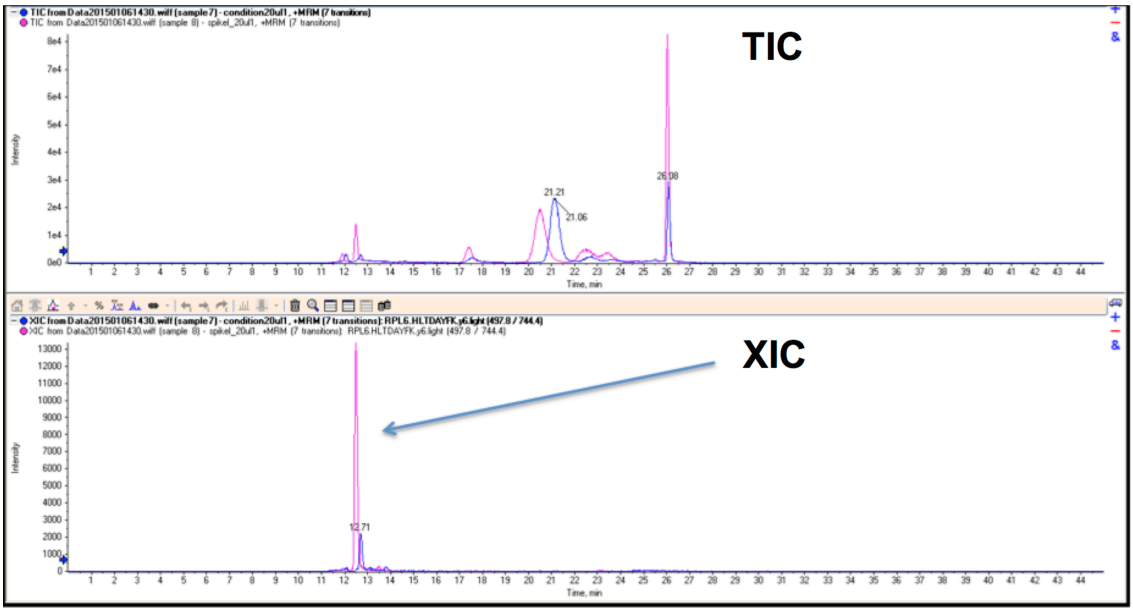
B. CANX, ^{78}GTLSGWILSK^{87}, m/z 531.3 ($M+2H^+$) → m/z 790.4



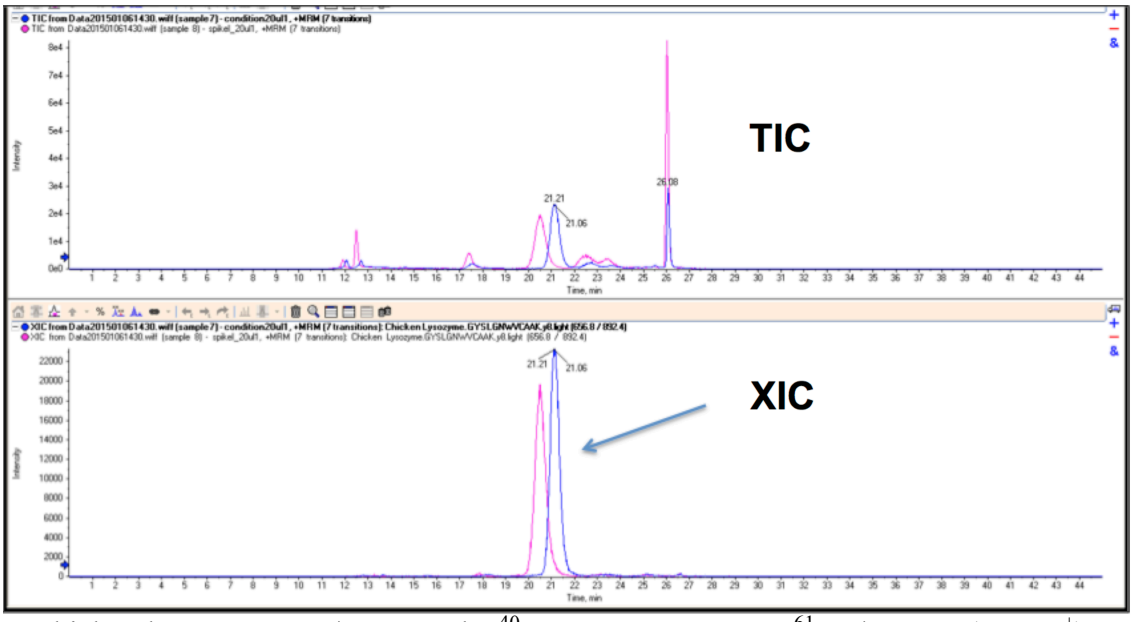
C. MSMP, ^{52}YFTLGESWLR^{61}, m/z 636.3 ($M+2H^+$) → m/z 747.4



D. SCP2, ^{48}IGGIFAFK^{55}, m/z 426.8 ($M+2H^{+}$) → m/z 739.4



E. RPL6, ^{211}HLTDAYFK^{218}, m/z 497.8 ($M+2H^{+}$) → m/z 744.4



F. chicken lysozyme, QA/QC sample, ^{40}GYSLGNWVCAAK^{61}, m/z 656.8 ($M+2H^+$) → m/z 892.4 (no synthetic chicken lysozyme peptide was spiked-in in the sample)

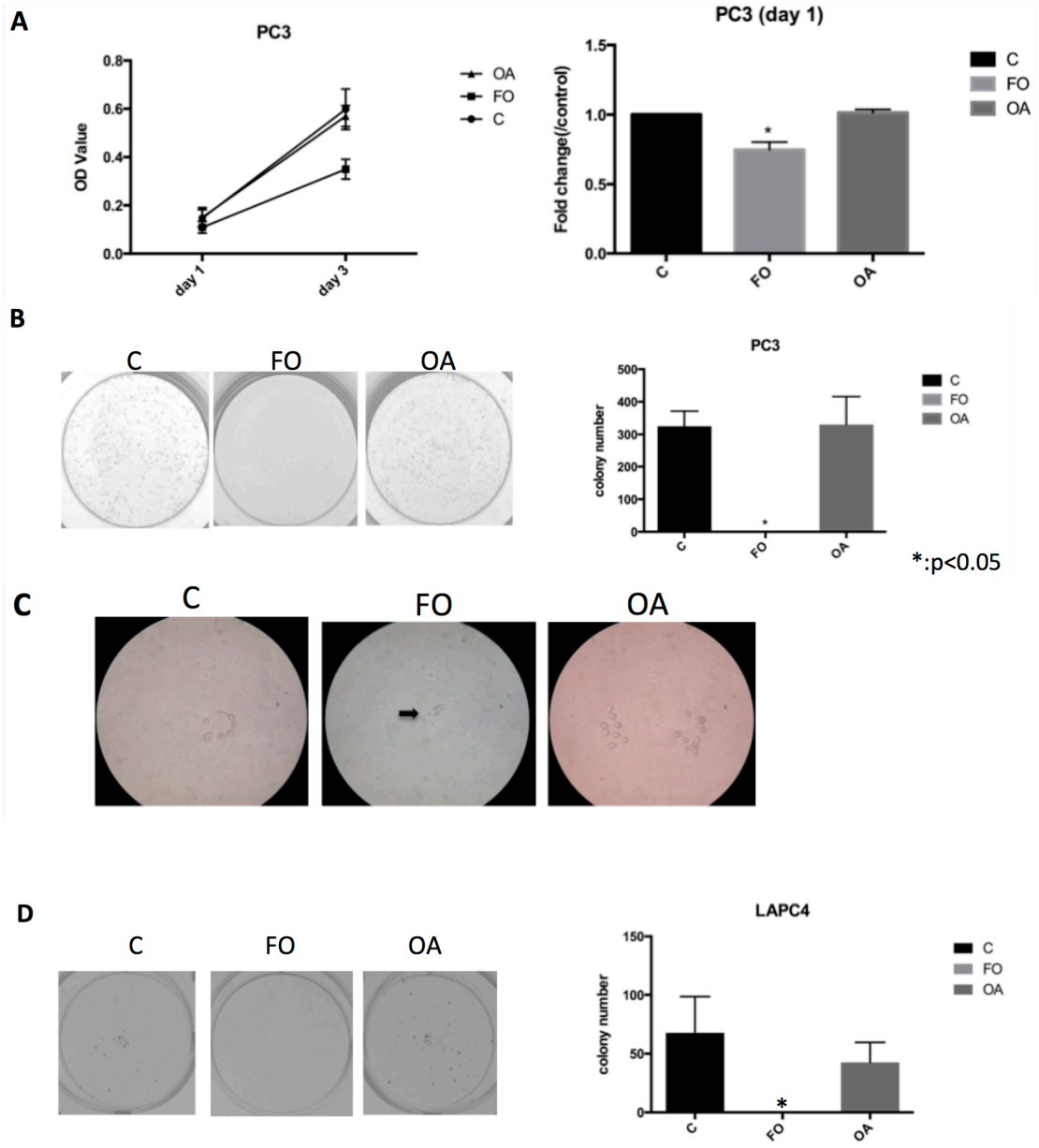
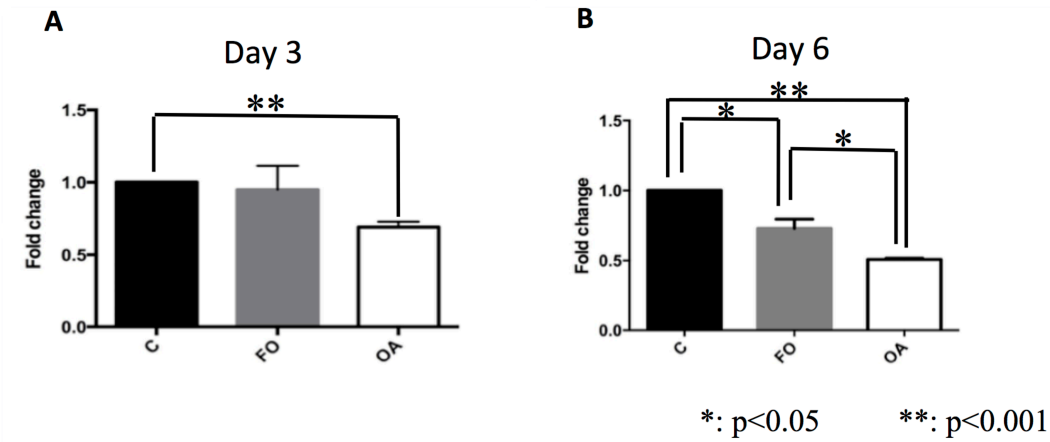


Figure 3-3 Effects of FO and OA on PCa cell viability.

A: MTT assay was measured 24-hr after PC3 cells were treated by control (0.1% ethanol), 100 μM fish oil or 100 μM oleic acid (n=3). **B:** PC3 cells were seeded at 1000 cell/well in 6-well plates, treated with control (0.1% ethanol), 100 μM FO, or 100 μM OA, respectively. Cells grew for approximately one week until the colonies were visible. Cells were fixed by methanol and stained with crystal violet (n=3). **C:** PC3 cell images captured during colony formation. **D:** Colony formation assay of LAPC4. LAPC4 cells were seeded at 3000 cell/well in 6-well plates. (n=3) *:p<0.05



*: $p<0.05$ **: $p<0.001$

Figure 3-4 Effects of FO and OA on FASN activity in PC3 cells.

Cells were seeded in 24-well cluster plates, treated with vehicle (0.1% ethanol), 100 μM FO or 100 μM OA for 3 days or 6 days. FASN activity was measured by ^{14}C-acetate incorporation assay, in which the scintillation counts were normalized by cell counts in parallel treated wells (n=3).

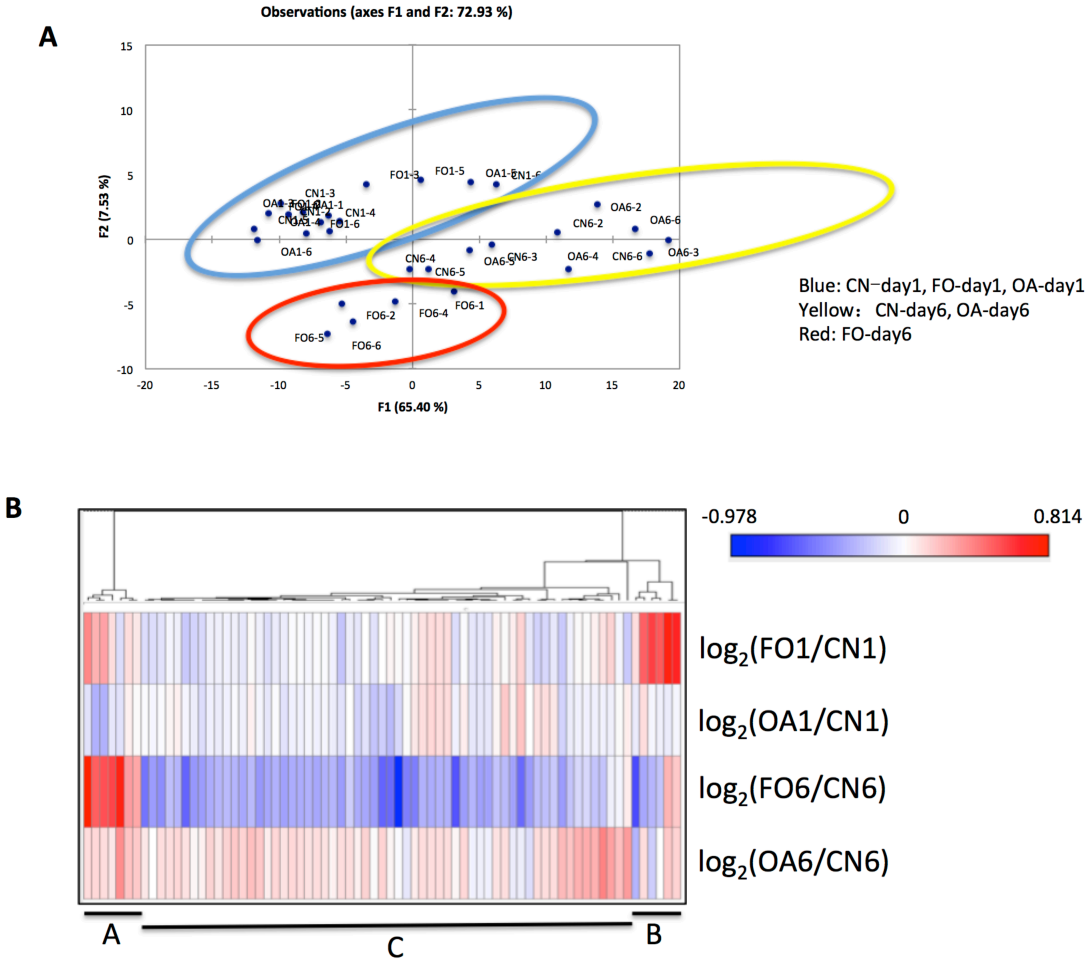
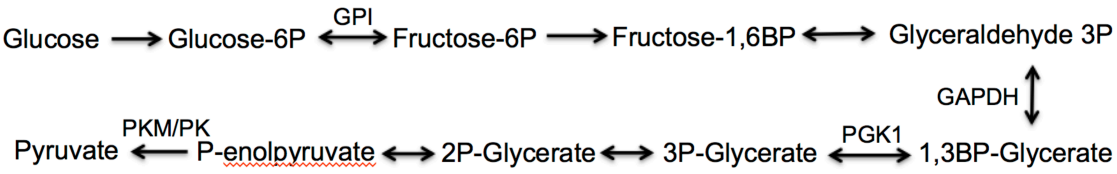


Figure 3-5 Principal component analysis (PCA).

A: PCA analysis of 127 significantly changed proteins (ANOVA $p \leq 0.05$, $q \leq 0.05$, n=5) shows graphic distribution of 30 samples (6 groups and 5 replicates in each) on axes of component 1 (F1) and component 2 (F2). **B:** By one minus Pearson Correlation, three major clusters were generated out of 73 proteins selected based on 127 proteins in (A) and has at least one significant change (t-test $p \leq 0.05$, n=5) in FO1/CN1 (fish oil vs. control, one-day), OA1/CN1 (oleic acid vs. control, one-day), FO6/CN6 (fish oil vs. control, six-day), or OA6/CN6 (oleic acid vs. control, six-day).



Genes	Protein name	Log2 FO/CN (day1)	Log2 OA/CN (day1)	Log2 FO/CN (day6)	Log2 OA/CN (day6)	ANOVA p value	q value
GPI	Glucose-6-phosphate isomerase	0.05 (NS)	0.03 (NS)	-0.22*	0.09 (NS)	7.22E-05	3.43E-03
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	0.00 (NS)	-0.04 (NS)	-0.32*	0.13 (NS)	2.00E-04	6.23E-03
PGK1	Phosphoglycerate kinase 1	0.01 (NS)	-0.10 (NS)	-0.33*	0.15 (NS)	2.00E-04	6.23E-03
PKM	Pyruvate kinase isozymes M1/M2	-0.04 (NS)	-0.05(NS)	-0.27*	0.04 (NS)	7.44E-05	3.43E-03
PK	Isoform M1 of Pyruvate kinase isozymes M1/M2	-0.05 (NS)	-0.06 (NS)	-0.26*	0.09 (NS)	2.00E-03	2.99E-02

Figure 3-6 Enzyme funtions in glycolysis/gluconeogenesis and its relative pathways are changed.

Enzyme functions in glycolysis/gluconeogenesis and its relative pathways are changed. Differentially expressed glycolysis/gluconeogenesis proteins in cluster C (*: t-test $p \leq 0.05$, n=5)

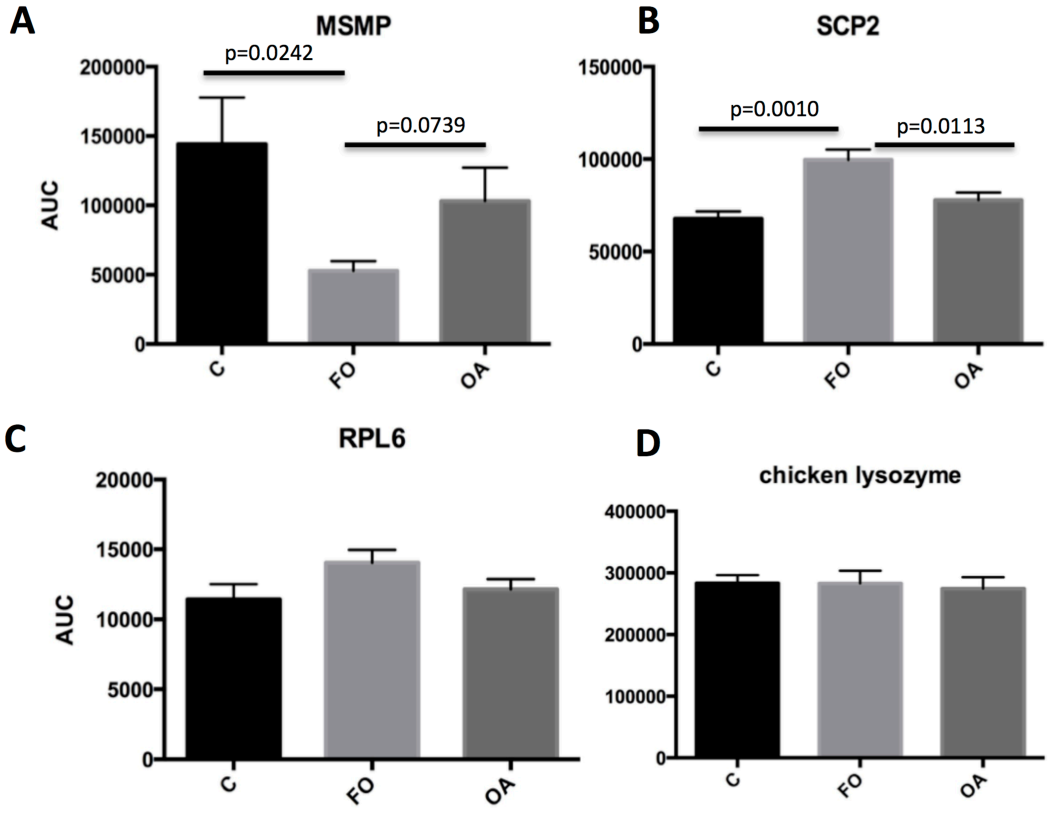


Figure 3-7 MRM validation of expression changes of MSMP and SCP2.

MRM validation of expression changes of MSMP **(A)**, SCP2 **(B)** in FO or OA group compared to control, with internal control RPL6 **(C)** and spiked-in external control chicken lysozyme **(D)** (n=6).

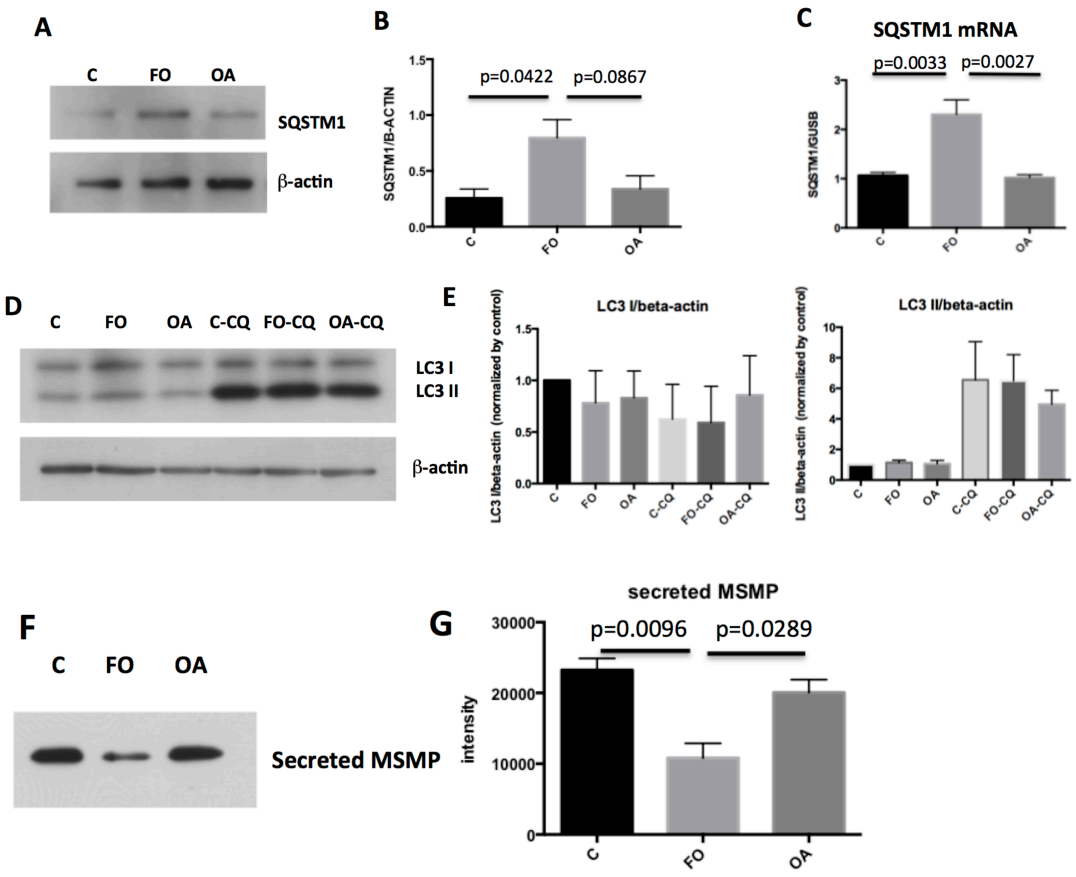


Figure 3-8 Western Blot validation of expression changes of MSMP, SQSTM1 and autophagy marker LC3 I/II in FO or OA group.

(A) SQSTM1 and β-actin; **(B)** Bar graph quantification of (A), n=3; **(C)** SQSTM-1 mRNA level by RT-PCR (GUSB mRNA serves as internal standard), n=5; **(D)** Western blot of LC3 I/II from cells treated with different FAs in the presence of 8 μM CQ for 1 day; **(E)** Bar graph quantification of (D), n=3; **(F)** secreted MSMP in medium; **(G)** Bar graph quantification of (F), n=3.

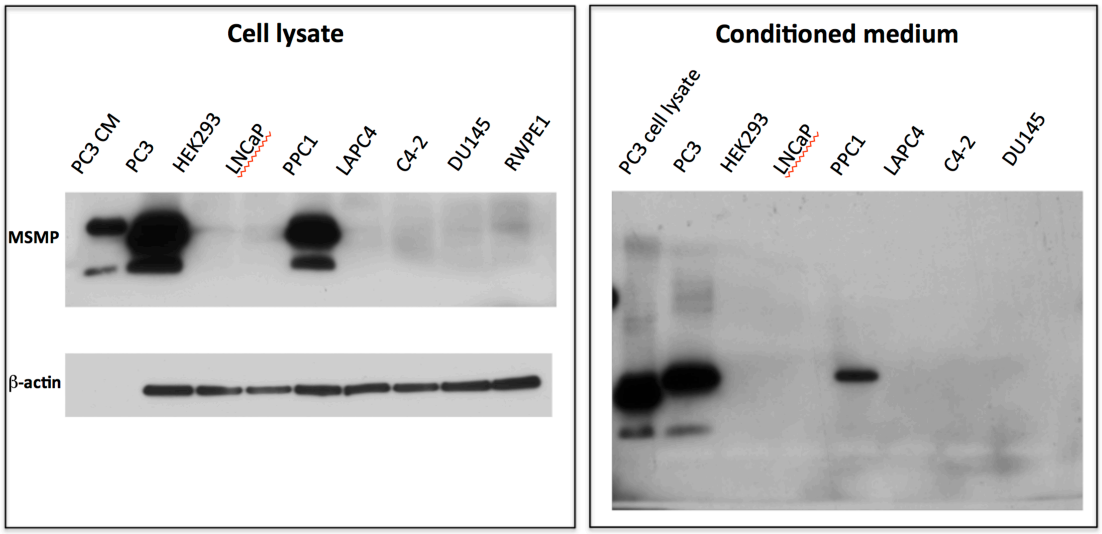


Figure 3-9 Expression of MSMP in other cell lines.

PC3 CM and lysate were used as a positive control.

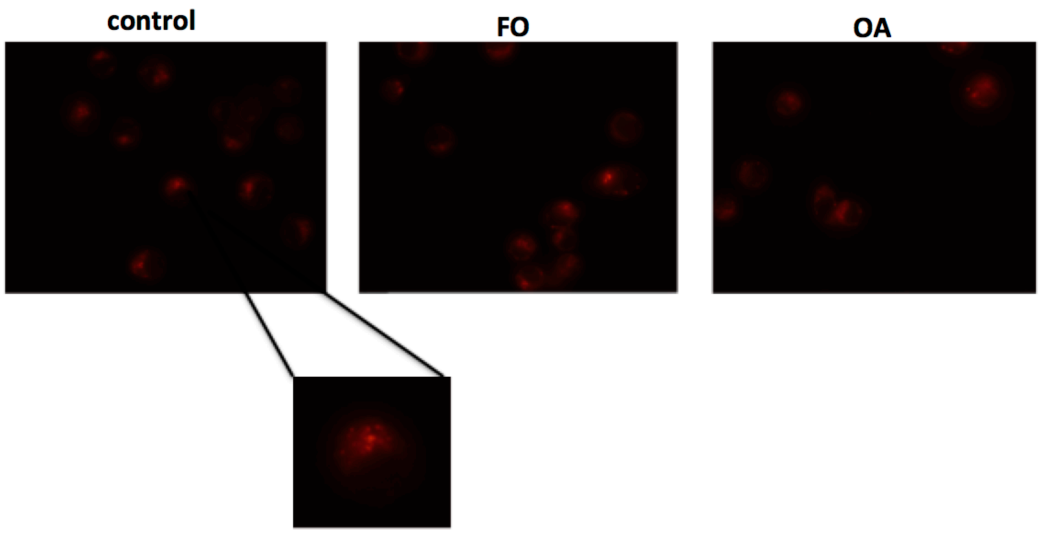


Figure 3-10 Proteasomal proteolysis activity detection by DQ-BSA under control, FO or OA treatment.

Chapter 4 Analysis of global phosphorylation changes induced by fish oil and oleic acid

1 Introduction

Cells respond to stress in various ways including the activation of survival pathways and the initiation of cell death. For cell growth and survival, it is neither safe nor efficient to modulate protein functions by solely relying on *de novo* protein synthesis. PTMs, especially phosphorylation and dephosphorylation, can regulate signaling pathways quickly and reversibly, and thus it might be a more efficient survival strategy than *de novo* synthesis of proteins. While the proteome profiling study provides an excellent opportunity to analyze the relative protein expression changes of thousands of proteins, the multitude of protein activities are primarily modulated by post-translational modifications, especially phosphorylation or dephosphorylation events. It is estimated that as many as one-third of all mammalian cellular proteins are phosphorylated, resulting in the regulation of a wide array of cellular processes [209]. Indeed, the initiation of many signaling pathways often begins with phosphorylation of cell surface receptors, such as growth factor receptors [210]. Thus, further insight into the complex signal transduction pathways using unbiased discovery approaches will aid in elucidation of the underlying biological impact of FO on prostate cancer cell function. In our previous global expression proteomics study [211], when prostate cancer cell line PC3 was treated with FO, cell viability suppression started as early as 24 hrs after the treatment. However, only a few proteins showed statistically significant changes in protein expression levels one day after the treatment. This led us to hypothesize that post-translational modifications, particularly phosphorylation or dephosphorylation events,

may play important roles in the metabolism of PUFAs in prostate cancer. In this study, a model prostate cancer cell line, PC3, was treated with 100 μM FO, 100 μM OA or vehicle. Cells were harvested 24-hr post-treatment and proteins were extracted, digested and enriched for phosphopeptides. An entire workflow of the study is shown in **Fig. 4-1**.

2 Materials and methods

1) Cell culture and fatty acid treatment

Prostate cancer cell line PC3 (ATCC, Manassas, VA) was cultured in RPMI medium (Hyclone, Logan, UT) with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO), 100 μg/ml streptomycin and 100 U/ml penicillin (Lonza, Walkersville, MD) and incubated in 5% CO_2 at 37°C. Fish oil (90% ethyl ester n-3 fatty acid: 40% DHA, 40%EPA and 10% other n-3 PUFA) and oleic acid (90% oleic acid ethyl ester) (KD Pharma Bexbach GmbH, Berlin, Germany) were diluted into ethanol, making 100 mM stocks, which were added into complete medium at 0.1%, with a 100 μM final fatty acid concentration. Cells were seeded in low density (170,000 cells per 100-mm culture dish) and treated with fatty acid the next day. Cells were harvested 24-hr post-treatment.

2) Cell lysis, protein reduction, alkylation and digestion

Cells were lysed (100 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100 and 5% glycerol), supplemented with 1% (v/v) phosphatase inhibitor (Sigma-Aldrich, St. Louis, MO) and 1% (v/v) protease inhibitor (Thermo-Fisher Scientific, Waltham, MA) (stock solution, 1 tablet into 1 mL H_2O). For each sample, 300 μg protein was taken for further sample prep and analysis, denatured with 50 mM TMAB containing 0.1% (w/v) RapiGest

surfactant (Waters, Milford, MA), followed by adding 10% (v/v) 1 M $(NH_4)_2CO_3$, pH=11. Reduction and alkylation were carried out by incubating with cocktail mix solution consisting of acetonitrile, iodoethanol and triethylphosphine (195:4:1, v/v). Digestion was performed by incubating overnight at 37°C with trypsin (Worthington Biochemical Corp, Lakewood, NJ) at a final concentration of 1:25 (w/w). To remove RapiGest, 1/9 volume of 1 M HCl was added to the sample to reduce pH to less than 3, then incubated at 37°C for 50 min, followed by centrifugation at 13,000 rpm. Supernatant that contains all peptides (phosphorylated and non-phosphorylated) was saved for next steps. Sample desalting was carried out through loading samples onto Silica C18 MacroSpin Columns (The Nest Group, Southborough, MA) followed by three washes in 5% ACN, 0.1% formic acid and finally eluted by 80% ACN, 0.1% formic acid. The eluent was concentrated in a speed vacuum concentrator (Genevac, NY) until dry.

3) Phosphopeptide enrichment

Polymer-based Metal-ion Affinity Capture PolyMAC-Ti was used for phosphopeptide enrichment. A PolyMAC-Ti agarose phosphopeptide enrichment kit was purchased from Tymora Analytical Operations (catalog#: AGARB, West Lafayette, IN) and enrichment was carried out according to manufacturer's suggested protocol. Briefly, dried sample peptides were first resuspended in loading buffer, mixed and incubated with PolyMAC-Ti reagent, then capture buffer was added into solution to adjust the pH between 5.5 and 6.5. The mixture of PolyMAC-Ti and peptides was transferred to capture gel containing a spin column and incubated in a shaker for 10 min. Non-binding peptides (non-phosphorylated) were discarded after spinning down the gel. The gel was incubated with loading buffer in shaker, washed twice with washing buffer and once with

deionized water. The phosphopeptides were eluted with elution buffer and dried in speed vacuum.

4) Global phosphoproteomics analysis

Data-dependent mass spectrometry (MS) analysis: Samples were reconstituted in 5% ACN with 0.1% formic acid in water, loaded onto a micro-flow column (1.0mm x 50mm, Tosoh Biosciences, King of Prussia, PA). The eluting flow rate was at 50 µl/min with a linear gradient developed from 3% to 80% ACN over 120 min. The eluents were directly sprayed into and analyzed by LTQ Orbitrap Velos Pro mass spectrometer (Thermo-Fisher Scientific, Waltham, MA). A full scan FTMS with m/z ranging between 350-2000; a follow-up ITMS scan was performed on the twelve top peaks selected. CID activation was done on precursor ion with normalized collision energy set at 35.0%. ESI conditions were set as follows: capillary temperature, 275°C; source heat temperature 40 °C, sheath gas flow, 20 arbi units; auxiliary gas flow, 0 arbi unit; ESI source voltage, 5 kV; the mass isolation width for CID was set at 2 m/z units.

Data analysis: The acquired MS data were searched in a batch mode against a UniProt human database (version: Human 20130403) using X!Tandem. Search parameters were set to locate differential modification of +79.96633 Da for phosphorylation. Precursor ion tolerance was 10 ppm and product ion tolerance was ± 1.0 Da. The identified peptides and proteins were validated through PeptideProphet and ProteinProphet in the Trans-Proteomics Pipeline (TPP, v.4.6). Peptides with probability >80% and proteins with probability over 90% are qualified for further analysis. IdentiQuantXL^{TM} [152] was applied to perform label-free quantification.

5) Western blot

Cells were harvested by scraping off the plate and cell pellets were lysed by lysis buffer consisting of 1% Triton-X100, 150 mM NaCl and 50 mM Tris, pH 7.6, supplemented with phosphatase inhibitor (Sigma-Aldrich, St. Louis, MO) and protease inhibitor (Thermo-Fisher Scientific, Waltham, MA). Protein concentration was measured by Bradford analysis. Cell lysates were denatured by boiling in 1 volume of 2 x Laemmli buffer (Bio-Rad, Hercules, CA) supplemented with 5% beta-mercaptoethanol (Fisher Scientific, Pittsburgh, PA). Proteins were loaded onto SDS-PAGE gel for separation and then transferred to PVDF membrane (Bio-Rad, Hercules, CA) at 100 mA for 120 min on ice. PVDF membranes were blocked with 5% BSA (Roche, Indianapolis, IN). Primary antibodies used were anti-phospho-PDHA1^{S232} (LifeSpan Biosciences, Seattle, WA), anti-phospho-PDHA1^{S293} (Emd Millipore Corporation, Billerica, MA), anti-phospho-PDHA1^{S300} (Emd Millipore Corporation), anti-PDHA1 (Cell Signaling Technology, Danvers, MA) and anti-alpha-tubulin (Sigma-Aldrich, St. Louis, MO). Secondary antibodies were also purchased from Cell Signaling Technology. ImageJ, an open source Java image processing program inspired by NIH Image, was used to quantify bands.

6) Pyruvate dehydrogenase (PDH) activity assay

PDH activity was measured using PDH Enzyme Activity Dipstick Assay kit (catalog#: ab109882, Abcam, Cambridge, MA), in which PDH is captured by an antibody immobilized on a dipstick. NADH produced by PDH reduces NBT to a colored precipitate. Protein concentration of the cell lysate is determined by BCA assay, and in each experiment, the same amount of protein is loaded and the protein amount used is within the linear range of detection verified in the testing experiment **(Fig. 4-2)**. Each

experiment was carried out in duplicate dipsticks. Band density was analyzed using ImageJ software. Unpaired t-test was performed in five biological replicates.

7) Bioinformatics and statics analysis

The gene ontology networks were extracted using Biological Networks Gene Ontology tool (BiNGO) in Cytoscape platform. Basic statistics (t-test, ANOVA and q-value) analyses were performed using Graphpad 6.0, Excel and R based q-value analysis website (www.qvalue.princeton.edu).

3 Results

1) Global phosphorylation changes induced by FO and OA

In this global phosphoproteomics discovery analysis, a total of 746 non-redundant phosphopeptides corresponding to 361 phosphoproteins were confidently identified with a FDR <1% **(Fig. 4-3A & 3B)**.

Observation frequency of certain phosphopeptides or phosphoproteins provides information of whether or not the phosphorylation is positively identified. Label-free quantification also allows for the relative abundance comparison among these identified phosphopeptides under different treatment conditions based on peak intensity quantification using AUC of the phosphopeptides. We evaluated the effects of both FO and OA on protein phosphorylation by combining and analyzing the values from both frequency and quantitation comparisons.

When all the phosphopeptides were compared among control, FO-treated and OA-treated groups, 215 non-redundant phosphopeptides representing 165

phosphoproteins were identified in all three groups **(Fig. 4-3A & 3B)**. When the FO-treated group and control group were compared, 194 phosphoproteins were in common. Thirty-seven other proteins were observed at least twice in the FO-treated group but not once in the control group, or at least twice in the control group but absent in the FO-treated group **(Table 4-1, List A)**. Likewise, the OA-treated group and the control group had 199 phosphoproteins in common and 22 other proteins were observed at least twice in the OA-treated group but not once in the control group, or at least twice in the control group but absent in the OA-treated group **(Table 4-1, List B)**.

Gene ontology (GO) distribution was also carried out using BiNGO plug-in application on the Cytoscape platform. This software provides visualization of overrepresented biological process categories after FDR corrections. BiNGO parameters in this study were set as follows: Hypergeometric test and Benjamini & Hochberg FDR correction were applied to the analysis; significant cut-off was set to $p \leq 0.05$; whole annotation was used as reference set and GO Biological Process was used as the annotation file. Protein list A, which was the phosphoprotein set differentially observed between the control and FO-treated groups, was analyzed using BiNGO. Thirty-two genes encoding these proteins were overrepresented in the subcategories mainly concerning metabolic processes, cellular responses to stress, and cell death **(Fig. 4-4)**. However, no enriched subcategories were overrepresented when using Protein list B, which is observed between the control and OA-treated groups.

Peak-intensity based label-free relative protein quantification was performed on 828 identified phosphopeptides, including some redundant phosphopeptides. The comparison was carried out among different treatment groups. As shown in **Fig. 4-5A**

and **5B**, when the FO-treated group was compared with the control and the OA-treated group, 80 and 47 phosphorylation level changes greater than two-fold were observed with statistical significance ($p \leq 0.05$, n=4), respectively. However, only a few minor changes were observed between the OA-treated and control groups **(Fig. 4-5C)**.

These observations indicate that FO induces more profound phosphorylation changes than OA, correlating well with cell viability suppression in prostate cancer cells one-day after the FO treatment from our previously published data [211].

2) Key protein phosphorylation altered under treatment of FO or OA

FO or OA modulation of cell signaling is suggested to be a multi-targeting and multi-level event. In this study, we used very stringent criteria to select phosphopeptides with significant changes, including: 1) at least one of the three treatment groups was negative for a particular phosphopeptide (peptide frequency=0); 2) At least one of the other two groups had a phosphopeptide frequency ≥0.5; 3) ANOVA test, $p \leq 0.05$, q-value≤0.05; 4) in one or more treatment group multiple comparison (corrected t-test), $p \leq 0.05$; and 5) fold-change ≥ 2. Based on these stringent criteria, 12 non-redundant phosphopeptides representing 12 phosphoproteins showed significant changes in phosphorylation level among these treatment comparisons (**Table 4-2 and Fig. 4-6**).

Although phosphorylation status may largely affect protein function [212] and many listed in **Table 4-2** have been reported in the previous large-scale global phosphoproteomic studies [213-215], very few have been either validated or studied for site-specific functions. In this study, one interesting observation is the elevated phosphorylation level of $SQSTM1^{S366}$ (fold-change=3.7, q=0.001). The phosphorylation site, serine-366, is located in the PEST domain [216], which is rich in proline (P),

glutamic acid (E), serine (S) and threonine (T) and considered to be associated with protein degradation [217]. However, whether the increased level of phosphorylation in this region is correlated with changes in SQSTM1 degradation remains to be investigated. It is worth noting that our previous study found up-regulation of SQSTM1 24-hr after the FO treatment at both the mRNA and protein levels [211], suggesting that the observed over-expressed protein level may not due to degradation.

Another interesting protein, pyruvate dehydrogenase alpha 1 (PDHA1), was also detected with an altered phosphorylation state. PDHA1 is a subunit of pyruvate dehydrogenase (PDH), which is a key enzyme involved in transforming pyruvate into acetyl-CoA and later being used in the tricarboxylic acid (TCA) cycle to carry out cellular respiration. Phosphorylation levels on PDHA1^{S232} is considered to be inversely associated with its enzyme activity [218]. From this global phosphoproteomics study, PDHA1^{S232} was significantly suppressed upon FO treatment, suggesting that FO may interfere with carbohydrate metabolism by enhancing PDH activity to restore the glycolysis-TCA cycle connection.

3) FO decreases phospho-PDHA1^{S232} and phospho-PDHA1^{S300} level and elevates PDH activity

Pyruvate dehydrogenase activity is inhibited by phosphorylation of PDHA1 and reactivated by dephosphorylation [218]. To validate our observation from the LC/MS analysis **(Fig. 4-7A**), an independent set of samples consisted of three biological replicates were analyzed by Western blot using an anti-phospho-PDHA1^{S232} antibody. As shown in **Fig. 4-7B**, more than 80% phosphorylation on phospho-PDHA1^{S232} was suppressed by FO as compared to control or OA-treated group ($p<0.05$). However, no

significant changes were observed between OA-treated and control groups (p=0.85) **(Fig. 4-7B and 4-7C)** and total PDHA1 was not affected by the fatty acid treatment **(Fig. 4-7B and 4-7D)**. Besides phospho-S232, two other phosphosites, phospho-S293 and phospho-S300 also participate in regulating PDHA1 activity. In global study, phospho-S293 was only detected in one injection and phospho-S300 was not observed. Western Blot results show that FO can also suppress phospho-S300 but phospho-S293 is not significantly changed **(Fig. 4-8)**. The PDH activity was measured using an antibody-based Dipstick assay. As shown in **Fig. 4-9A and 4-9B**, PDH activity was significantly elevated by FO as compared to OA-treated group.

4) Global phosphorylation changes induced by prolonged treatment of FO and OA

Given that global proteomics study in prolonged FA treated PC3 cells reveals multiple molecular targets of FO, global phosphoproteomics discovery analysis was also performed in PC3 cells, which are treated for 6-days. A total of 470 non-redundant phosphopeptides corresponding to 276 phosphoproteins were confidently identified with a FDR <1% **(Fig. 4-10A & 10B)**. 117 phosphopeptides and 116 phosphoproteins were observed in all three treatments. Quantitative analysis showed that after 6 days FO treatment, only two phosphopeptides with over two-fold change are significant compared to control ($p \leq 0.05$, n=4). Noticeably, all the significantly changed phosphopeptides in OA groups (compared to either FO or control groups) are down-regulated **(Fig. 4-11A & 11B).**

Given that in this day-6 data set, no phosphopeptides have ANOVA test q value$\leq$0.05, we used less stringent selection criteria than day-1 data set, including: 1) at least one of the three treatment groups was negative for a particular phosphopeptide

(peptide frequency=0); 2) At least one of the other two groups had a phosphopeptide frequency ≥0.5; 3) ANOVA test, p≤0.05; 4) in one or more treatment group multiple comparison (corrected t-test), p≤0.05; and 5) fold-change ≥ 2. Based on these criteria, 6 non-redundant phosphopeptides representing 6 phosphoproteins showed significant changes in phosphorylation level among these treatment comparisons (**Table 4-3**).

4 Discussion

Based on the data from our previous global expression profiling experiments, only a few proteins showed altered expression when control, FO-treated, and OA-treated groups were compared 24-hr post-treatment, implicating PTMs may play an important role in transmitting n-3 PUFA signals. In this study, our goal was to globally profile the phosphoproteome to help understand how FO affects cellular functions of prostate cancer cells through regulating phosphorylation. There have been studies suggesting that the phosphoproteome changes upon fatty acid stimulation [219, 220]. However, these previous studies were performed in yeast and no global phosphoproteome studies on fatty acid modulation of phosphoproteins have been carried out in mammalian cells. This is the first study we are aware of that elucidates the global protein phosphorylation alterations with n-3 PUFA treatment in prostate cancer cells using a modern high-throughput LC/MS platform.

Among the proteins with phosphopeptide levels decreased by FO treatment, several have been well studied. RRM2 is one of the enzymes responsible for DNA synthesis by turning ribonucleotides to deoxyribonucleotides. $RRM2^{S20}$ phosphorylation can be triggered by p34cdc2 kinase but mutations at Serine-20 cannot induce enzyme

activity alterations [221]. Another study showed that in addition to DNA synthesis functions, RRM2 can also inhibit the canonical Wnt/beta-catenin pathway and Wnt3a can phosphorylate $RRM2^{S20}$ to reduce RRM2's suppression of the Wnt pathway [222]. Another protein, MCM3, functions in DNA replication and plays an important role in cell cycle checkpoint [223]. $MCM3^{S711}$ and $MCM3^{T722}$ were reported to be phosphorylated by cyclin E/Cdk2 in 293T cells [224]. The observed phosphorylation alterations in both RRM2 and MCM3 by FO may suggest a cell cycle suppression induced by FO. The other two proteins among these down-phosphorylated proteins, SRRM1 and SRRM2, are members of the protein complex participating in pre-mRNA processing [225] and we suggest that they may be involved in rapid cell response to adjust biosynthesis under oxidative stress.

Among the proteins with phosphopeptide levels elevated by FO treatment, AHNAK has been reported to induce arachidonic acid-dependent activation of phospholipase C-gamma1 [226]. Arachidonic acid is a n-6 PUFA, however, no studies to date have evaluated AHNAK functions with n-3 PUFA treatment. Another over-phosphorylated protein, DYNC1LI1, is a component of the cytoplasmic dynein 1 complex, which helps microtubule dependent cargo trafficking [227]. Thus FO may induce changes in intracellular trafficking through function of DYNC1LI1. Interestingly, DYNC1LI1 (cytoplasmic dynein 1) is also reported to interact with SQSTM1 [228], in which Ser-366 is up-regulated by FO. SQSTM1 is a multi-functional protein, which also plays a critical role in polyubiquitinated substrate degradation during autophagy [229]. It is known that SQSTM1 itself is subjected to degradation in autophagy process [230], and Ser-366 locates to the PEST domain [216, 217], which may be associated with

degradation. Whether the elevation of phospho-SQSTM1^{S366} is related to FO induced suppression of cell viability deserves further investigation.

One important discovery from this study is down-regulation of phospho-PDHA1^{S232}, which may be a critical target of FO action. PDH complex is composed of three enzymes: E1 (PDH), E2 (dihydrolipoyl transacetylase or DLAT), and E3 (dihydrolipoyl dehydrogenase or DLD) [231]. The E1 alpha subunit contains the E1 active site and plays a key role in the function of the PDH complex, which connects glycolysis with tricarboxylic acid (TCA) cycle by converting pyruvate to acetyl-CoA and CO_2. It is already known that pyruvate dehydrogenase kinases and pyruvate dehydrogenase phosphatases (PDPs) regulate phosphorylation of PDHA1^{S232}, PDHA1^{S293}, and PDHA1^{S300} [232], which can also be induced by different chemotherapeutic drugs and UV treatment [233]. Western blot results showed that not only serine232 but also serine300 is downregulated by FO treatment, indicating that FO may regulate expression or activity of pyruvate dehydrogenase kinases or PDPs in PCa cells.

As previously suggested by many studies, PDH activity is inhibited by phosphorylation of PDHA1 [218, 231, 234]. Consistent with other studies, we also observed an enhanced PDH activity when phospho-PDHA1^{S232} and phospho-PDHA1^{S300} are down-regulated (**Figs. 4-8 & 4-9**). A feature that most cancers adopt is altered metabolism often referred to as the Warburg effect that is characterized by accelerated aerobic glycolysis with an absence of the pyruvate product oxidation in mitochondria. Although, unlike other cancers, the main bioenergy source for prostate cancer cells are fatty acids instead of glucose [93], there is evidence indicating that glycolysis enzyme

expression is higher in prostate and many other cancers [235]. Several of the enzymes involved in glycolysis are also critical regulators of apoptosis and gene transcription, implicating that links between metabolic regulators, apoptosis, and gene transcription are established directly through the enzymes that control metabolism [236]. It is therefore not so surprising that glycolytic enzymes are suppressed by PUFAs [202, 203], and inhibition of glycolysis can elevate prostate cancer cell sensitivity toward stress [201]. Our data suggest that FO might help partially restore TCA cycle function and normalize glucose metabolism in prostate cancer cells. It is still unknown whether PDH activity induced by FO is connected to suppression of cell viability observed in our previous global expression study [211]. One report suggested that the elevated expression and activity of PDH are associated with inhibition of cell viability in other cancer cell lines [237] and inhibiting the pyruvate dehydrogenase kinases (e.g., by dichloroacetate) increases the flux of pyruvate into the mitochondria, promoting glucose oxidation over glycolysis [238]. As the result, down-regulation of phosphorylation on PDH subunits suppresses tumor growth [238]. On the contrary, n-3 PUFAs have been suggested to increase pyruvate dehydrogenase kinase expression at the transcriptional level in muscle [239] and dendritic cells [240]. Whether PDH dephosphorylation participates in FO induced cell viability inhibition and how FO regulates pyruvate dehydrogenase kinases or PDPs still need to be investigated.

Although OA does not seem to inhibit cell viability while FO does [211], it does alter a few protein phosphorylation changes in this global phosphoproteomic study (**Fig. 5-4C**). OA is known to inhibit VEGFR-1 autophosphorylation [241] and to induce ERK2

phosphorylation [242]. However, as compared to FO, OA showed much lower degree of activity in altering protein phosphorylation in prostate cancer cells.

We present a large-scale global phosphoproteome analysis that utilized state-of-the-art technologies. Using an established cell line model, we show that phosphorylation changes induced by PUFAs can be quantitatively monitored by sensitive mass spectrometric methods. Validation of phospho-PDHA1^{S232} levels by Western blot and assessment of phospho-PDHA1^{S300} level and PDH activity by a Dipstick assay also provide us increased confidence that metabolic-targeting approaches may be therapeutically valuable for prostate cancer management and prevention. Additional studies investigating whether pyruvate dehydrogenase kinase, as well as identifying whether other PDPs are involved in pathway regulation by FO.

Table 4-1 Phosphoproteins differentially detected in fatty acid treated groups by observation frequency.

List A: Phosphoprotein level change observed twice or more in the FO-treated group but absent in the control group, or twice or more in the control group but absent in the FO-treated group.

Protein ID	Gene Name	Protein Name	Protein Frequency (Group C)	Protein Frequency (Group F)	Protein Frequency (Group OA)
H3BV87	ARMCX3	Armadillo repeat-containing X-linked protein 3 (Fragment)	0	0.5	0
E7ESP2	CDK11A	Cyclin-dependent kinase 11A	0.5	0	0.5
P51397	DAP	Death-associated protein 1	0.5	0	0.25
E9PHI6	DYNC1LI1	Cytoplasmic dynein 1 light intermediate chain 1	0	0.5	0
E9PC74	EIF2B5	Translation initiation factor eIF-2B subunit epsilon	0.5	0	0.25
Q14315	FLNC	Filamin-C	0.5	0	0.25
C9J0Y3	GOLGA4	Golgin subfamily A member 4 (Fragment)	0	0.5	0
E7EVX2	GOLGA4	Golgin subfamily A member 4 (Fragment)	0	0.5	0
Q96RR5	HCA90	Hepatocellular carcinoma-associated antigen 90	0	0.75	0
P10809	HSPD1	60 kDa heat shock protein, mitochondrial	0.5	0	0.75
O43719	HTATSF1	HIV Tat-specific factor 1	0.5	0	0.25
H0Y5W0	HUWE1	E3 ubiquitin-protein ligase HUWE1 (Fragment)	0.75	0	0.25
P11717	IGF2R	Cation-independent mannose-6-phosphate receptor	0	1	0.5
B4DTU4	LIG1	DNA ligase	0.5	0	0
E9PLH4	LMO7	LIM domain only protein 7	0.5	0	0.75
E9PMP7	LMO7	LIM domain only protein 7 (Fragment)	0.5	0	0.75
E9PMS6	LMO7	LIM domain only protein 7	0.5	0	0.75
H0Y424	LMO7	LIM domain only protein 7 (Fragment)	0.5	0	0.75
C9J013	MCM2	DNA replication licensing factor MCM2 (Fragment)	0.5	0	0.25
H0Y9Z8	NCAPG	Condensin complex subunit 3 (Fragment)	0.5	0	0.25
Q9ULX3	NOB1	RNA-binding protein NOB1	0.5	0	0.5
C9JIG9	OXSR1	Serine/threonine-protein kinase OSR1	0	0.5	0
E9PBS1	PAICS	Phosphoribosylaminoimidazole carboxylase (Fragment)	0	1	0.5
P08559	PDHA1	Pyruvate dehydrogenase E1 component subunit alpha, somatic form, mitochondrial	0.75	0	0.5
Q15124	PGM5	Phosphoglucomutase-like protein 5	0.5	0	0
E9PJ24	PHRF1	PHD and RING finger domain-	0.5	0	0

		containing protein 1			
H3BN34	PKM	Pyruvate kinase isozymes M1/M2 (Fragment)	0	0.75	0
Q9NS91	RAD18	E3 ubiquitin-protein ligase RAD18	0.5	0	0
D6RAD2	RFC1	Replication factor C subunit 1 (Fragment)	0.5	0	0.5
C9J0A5	RNF20	E3 ubiquitin-protein ligase BRE1A (Fragment)	1	0	0
P31350	RRM2	Ribonucleoside-diphosphate reductase subunit M2	0.5	0	0.25
Q13501	SQSTM1	Sequestosome-1	0	0.75	0.25
F6XM96	TFIP11	Tuftelin-interacting protein 11 (Fragment)	0	0.5	0
E7ERH3	TNS3	Tensin-3	0.5	0	0.75
A6NNK5	TP53BP1	Tumor suppressor p53-binding protein 1	0.75	0	0
C9JXV0	TP53BP1	Tumor suppressor p53-binding protein 1 (Fragment)	0.75	0	0
O43399	TPD52L2	Tumor protein D54	0	0.5	0

Table 4-1:

List B: Phosphoprotein level change observed twice or more in the OA-treated group but absent in the control group, or twice or more in the control group but absent in the OA-treated group.

Protein ID	Gene Name	Protein Name	Protein Frequency (Group C)	Protein Frequency (Group F)	Protein Frequency (Group OA)
B4E0G4	PHF6	PHD finger protein 6	0	0.25	0.5
B7Z8E4	CCNY	Cyclin-Y	0	0	0.5
E9PBS1	PAICS	Phosphoribosylaminoimidazole carboxylase (Fragment)	0	1	0.5
F5GYR8	USO1	General vesicular transport factor p115	0	0	0.5
F5H7B0	SKIV2L	Helicase SKI2W	0	0	0.75
P11717	IGF2R	Cation-independent mannose-6-phosphate receptor	0	1	0.5
Q8WW12	PCNP	PEST proteolytic signal-containing nuclear protein	0	0	0.5
Q92625	ANKS1A	Ankyrin repeat and SAM domain-containing protein 1A	0	0	0.5
A6NNK5	TP53BP1	Tumor suppressor p53-binding protein 1	0.75	0	0
B4DTU4	LIG1	DNA ligase	0.5	0	0
C9J0A5	RNF20	E3 ubiquitin-protein ligase BRE1A (Fragment)	1	0	0
C9JXV0	TP53BP1	Tumor suppressor p53-binding protein 1 (Fragment)	0.75	0	0
E9PJ24	PHRF1	PHD and RING finger domain-containing protein 1	0.5	0	0
H3BQ34	PKM	Pyruvate kinase	0.75	0.75	0
H3BUF6	ATXN2L	Ataxin-2-like protein	0.5	0.25	0
H7C3D5	FAM134A	Protein FAM134A (Fragment)	0.5	1	0
O00505	KPNA3	Importin subunit alpha-3	0.75	0.25	0
O95218	ZRANB2	Zinc finger Ran-binding domain-containing protein 2	0.5	0.25	0
P51580	TPMT	Thiopurine S-methyltransferase	0.5	0.5	0
Q15124	PGM5	Phosphoglucomutase-like protein 5	0.5	0	0
Q5VWV2	PARD3	Partitioning defective 3 homolog	0.5	0.25	0
Q9NS91	RAD18	E3 ubiquitin-protein ligase RAD18	0.5	0	0

Table 4-2 Key phosphopeptides with significant changes 24 hours post-treatment.

Protein ID	Gene Name	Protein Name	Peptide Sequence	Peptide Frequency (Group C)	Peptide Frequency (Group F)	Peptide Frequency (Group OA)	FC_F_C	FC_OA_C	FC_OA_F	ANOVA_p_value	q-value
P31350	RRM2	Ribonucleoside-diphosphate reductase subunit M2	VPLAPITDPQQLQLSPLK	0.5	0	0.25	-7.8	-1.8	4.3	0.0009	0.009
P08559	PDHA1	Pyruvate dehydrogenase E1 component subunit alpha, somatic form, mitochondrial	YGMGTSVER	0.75	0	0.5	-3	-1.2	2.4	0.001	0.009
E9PLH4	LMO7	LIM domain only protein 7	ATLSSTSGLDLMSESGEGEISPQR	0.5	0	0.75	-2.6	-1.2	2.2	0.002	0.014
F5GXF7	ZNF185	Zinc finger protein 185	RESCGSSVLTDFEGKDVATK	0.25	0	1	-1.8	1.2	2.2	0.01	0.030
B4DWW4	MCM3	DNA replication licensing factor MCM3	DGDSYDPYDFSDTEEEMPQVHTPK	0.25	0.5	0	-2.3	-1.3	1.7	0.007	0.030
Q9ULX3	NOB1	RNA-binding protein NOB1	KDDSDDDGGGWITPSNIK	0.25	0	0.5	-2.4	-1.5	1.6	0.001	0.009
Q9UQ35	SRRM2	Serine/arginine repetitive matrix protein 2	CRSPGMLEPLGSSR	0	0.25	0.5	-2.1	-1.4	1.5	0.004	0.023
Q8IYB3	SRRM1	Serine/arginine repetitive matrix protein 1	KPPAPPSPVQSQSPSTNWSPAVPVKK	0.5	0	0	-2.6	-2.5	1.1	8.00E-06	0.001
Q9Y6G9	DYNC1LI1	Cytoplasmic dynein 1	KPVTVSPTTPTSPTEGEAS	0	0.5	0	2	-1.2	-2.5	3.00E-05	0.001

		light intermediate chain 1									
Q09666	AHNAK	Neuroblast differentiation-associated protein AHNAK	DDGVFVQEVTQNSPAAR	0	0.75	0	2.2	-1.3	-2.8	0.0003	0.006
P22234	PAICS	Multifunctional protein ADE2	TKEVYELLDSPGK	0	0.75	0	2.2	-1.3	-2.8	0.002	0.014
Q13501	SQSTM1	Sequestosome-1	EVDPSTGELQSLQMPESEGPSSLDPSQEGPTGLK	0	0.75	0.25	3.7	1	-3.6	2.00E-05	0.001

(FC: Fold Change)

Table 4-3 Key phosphopeptides with significant changes 6-days post-treatment

Protein ID	Gene Name	Protein Name	Peptide Sequence	Peptide Frequency (Group C)	Peptide Frequency (Group F)	Peptide Frequency (Group OA)	FC_FC	FC_OA_C	FC_OA_F	ANOVA_p_value
Q13442	PDAP1	28 kDa heat- and acid-stable phosphoprotein	SLDSDESEDEEDDYQQK (Ser60)	0	0.5	0	1.8	-1.4	-2.6	0.03
Q52LW3	ARHGAP29	Rho GTPase-activating protein 29	SFENVSVESVDSSSEK (Ser171)	0	0.5	0	1.4	-2.4	-3.3	0.009
Q5H9R7	PPP6R3	Serine/threonine-protein phosphatase 6 regulatory subunit 3	IQQFDDGGSDEEDIWEEK (Ser617)	0	0.5	0	1.2	-3.5	-4	0.02
Q15459	SF3A1	Splicing factor 3A subunit 1	FGESEEVEMEVESDEEDDKQEK (Ser329)	0.25	0	0.75	1.1	-2.3	-2.6	0.03
Q86V48	LUZP1	Leucine zipper protein 1	EKPDSDDDLDIASLVTAK (Ser659)	0	0.5	0	-1.3	-3.1	-2.3	0.04
Q9BQE3	TUBA1C	Tubulin alpha-1C chain	DYEEVGADSADGEDEGEEY (Ser439)	0	0.5	0	1.4	-2.7	-3.8	0.007

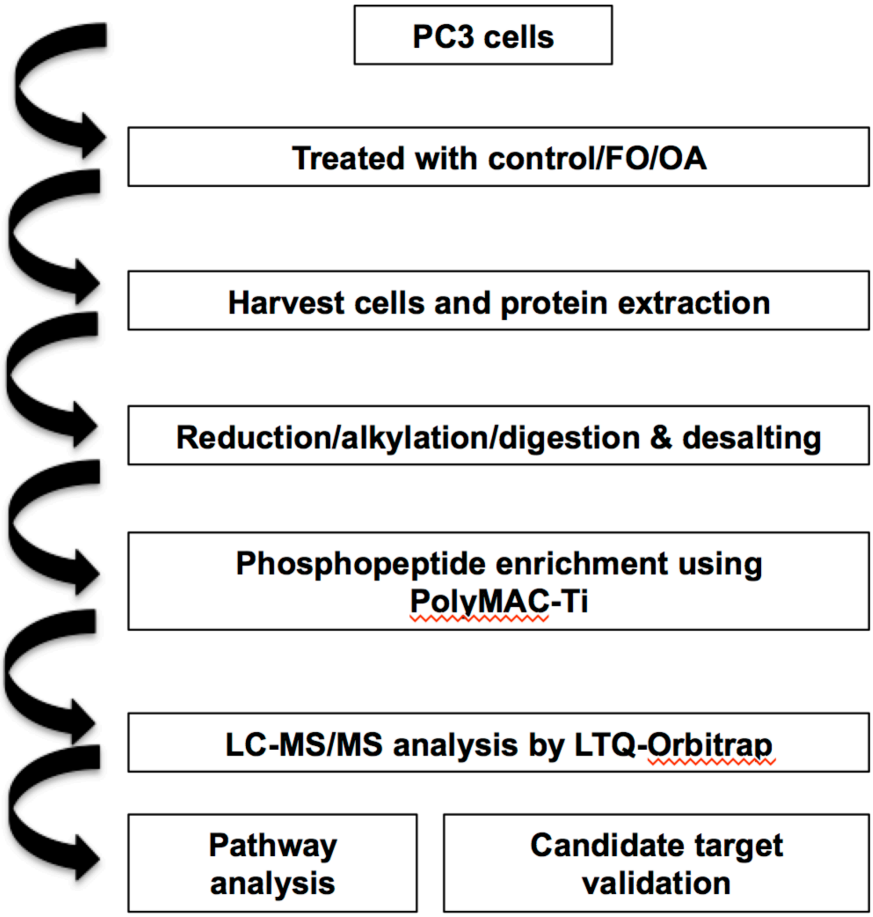


Figure 4-1 Work-flow of the global phosphoproteomics study.

PC3 cells were plated at 170K cells per 100 mm dish. After cells were attached for one day, culture medium was replaced with fresh medium supplemented with 100 μM FO, OA, or only vehicle (0.1% ethanol). Cells were harvested after 24 hours treatment. Protein was extracted and phosphopeptides were enriched using Polymac-Ti kit before LC/MS/MS analysis.

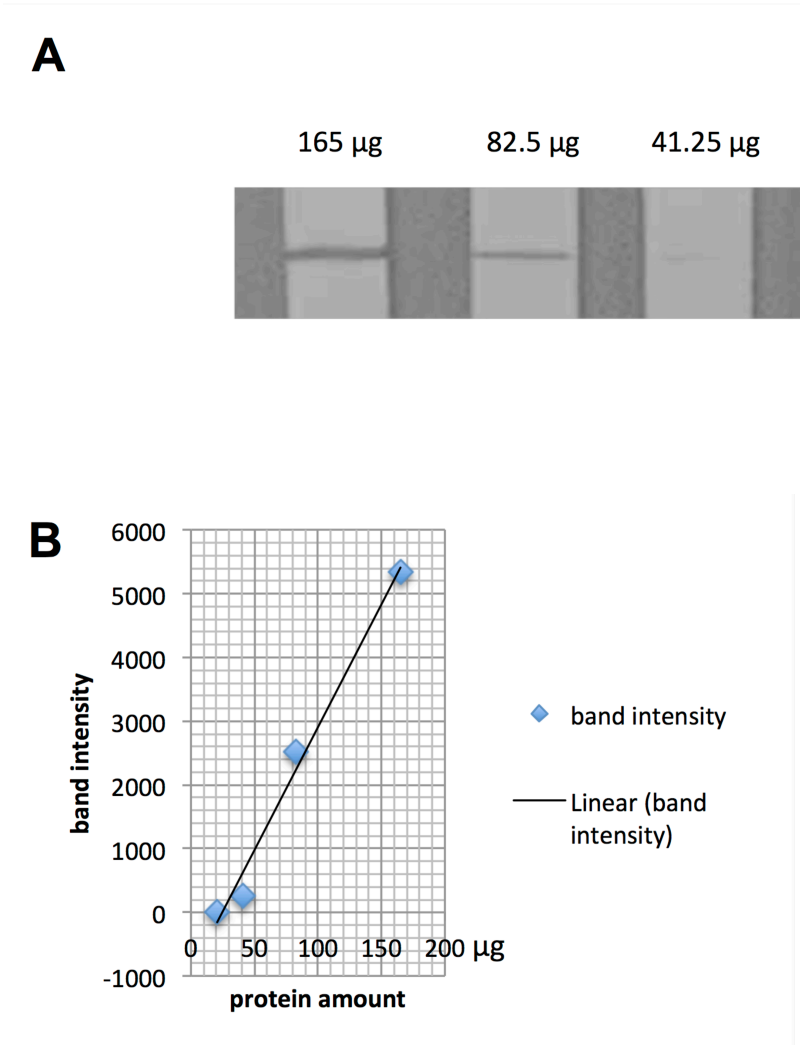


Figure 4-2 Linear range of PDH activity Dipstick assay.

A: 165 µg, 82.5 µg, 41.25 µg, 20.6 µg protein in PC3 lysate were loaded on each dipstick.
B: Linear regression was performed based on the protein amount on each dipstick and its corresponding band intensity.

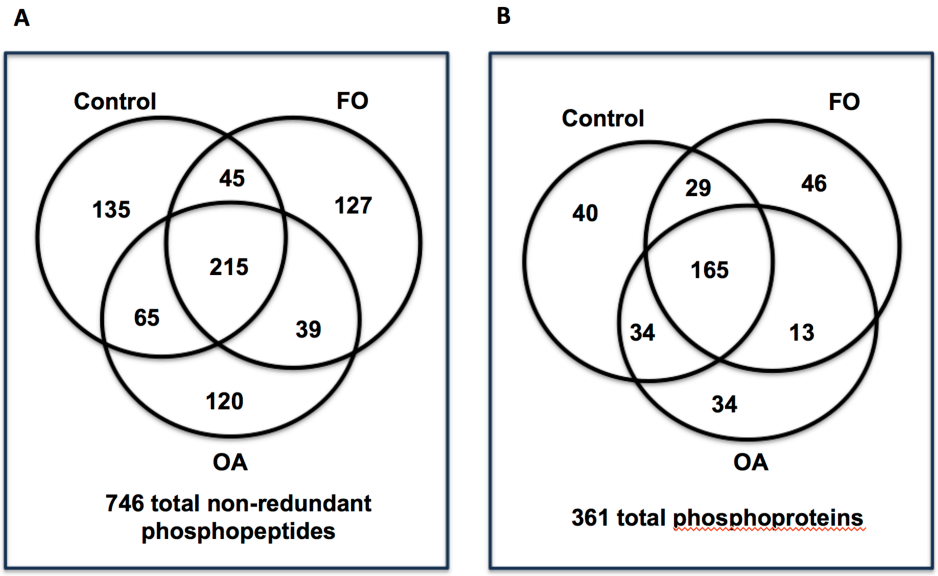


Figure 4-3 Venn diagrams of identified phosphopeptides and corresponding phosphoproteins.

A: Number of non-redundant phosphopeptide identified in at least 1 of 4 replicates under each condition. **B**: Number of corresponding phosphoproteins.

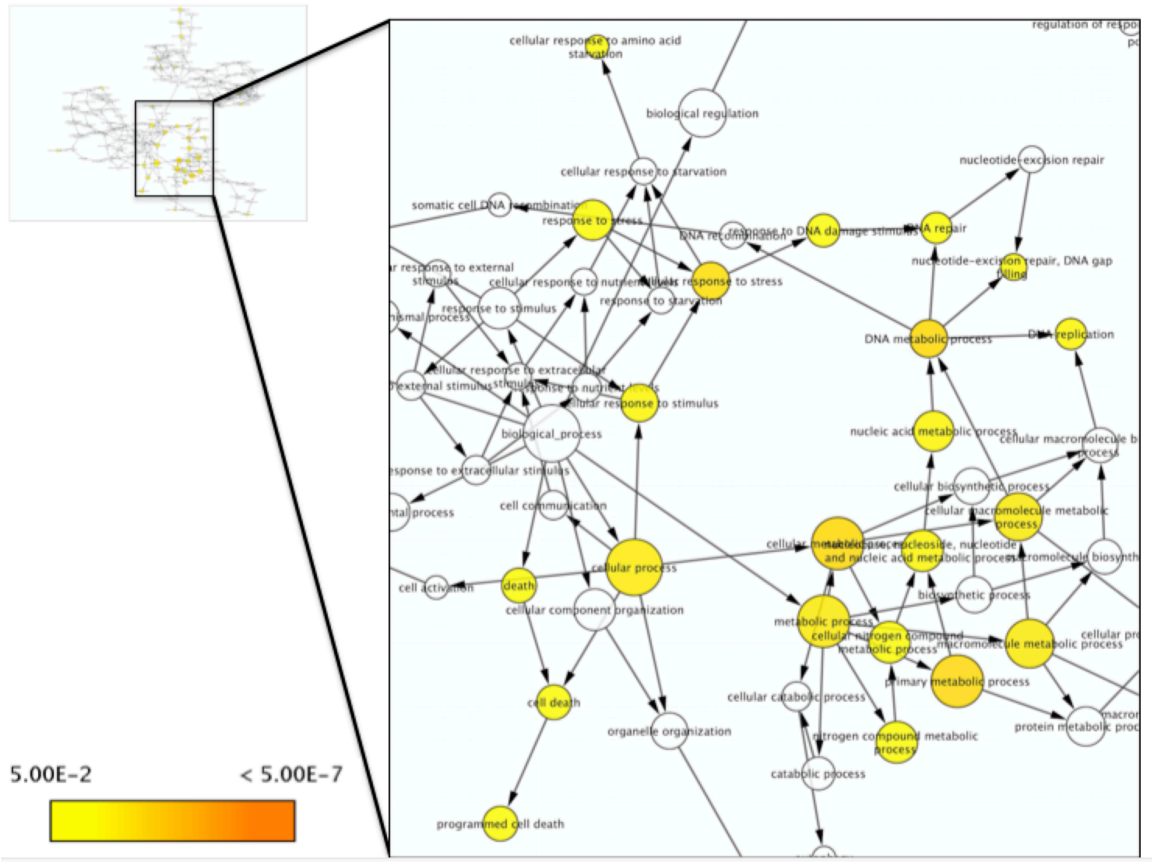


Figure 4-4 Biological network enrichment analysis of genes encoding differentially expressed phosphoproteins between control and FO treated cells.

A gene list consisting of 32 genes was generated from 37 phosphoproteins with different phosphorylation levels with FO group and control comparisons. Yellow circles represent significant gene ontology categories overrepresented by gene sets with different p values. Analysis was performed using BiNGO in Cytoscape platform.

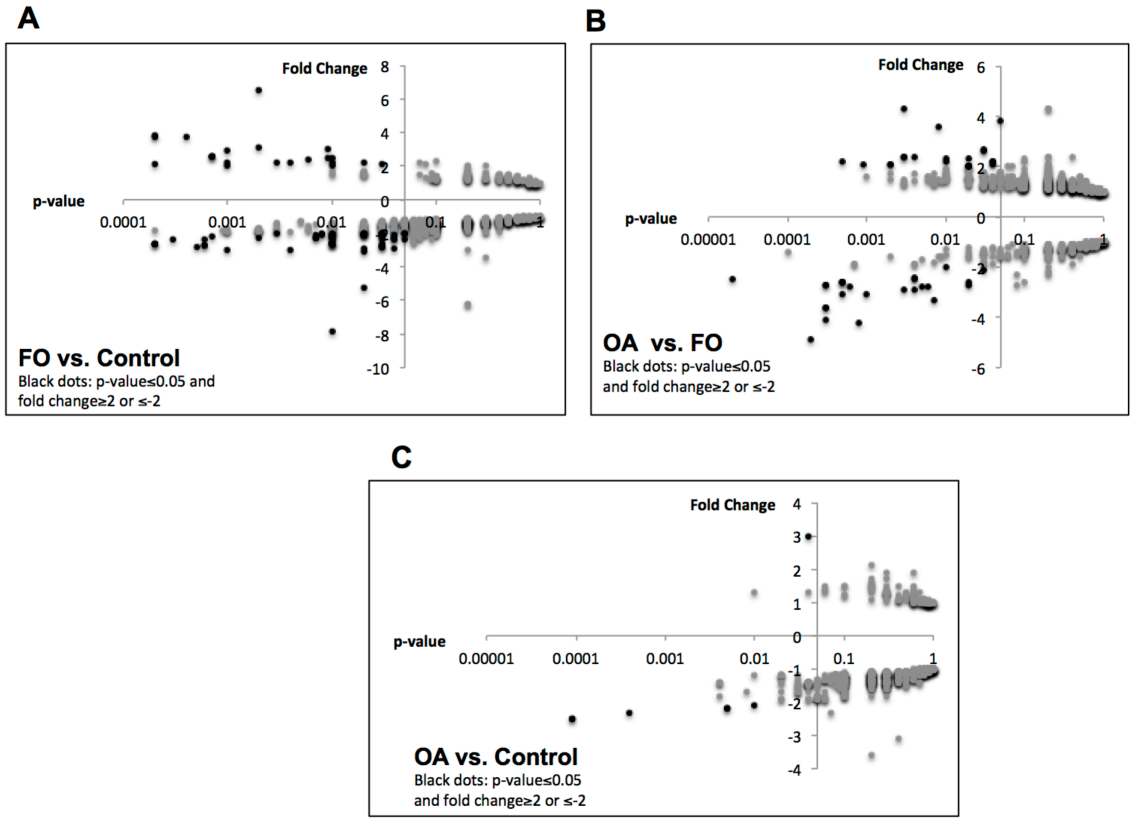


Figure 4-5 Relative comparisons of phosphopeptide levels based on label-free quantification.

Fold change (y-axis) and t-test p-value (x-axis) were presented for each phosphopeptide (n=828). P-value axis crosses fold change axis at p=0.05. Relative changes with p-value≤0.05 and fold change≥2 or ≤-2 were labeled in each figure by black dots (n=4).

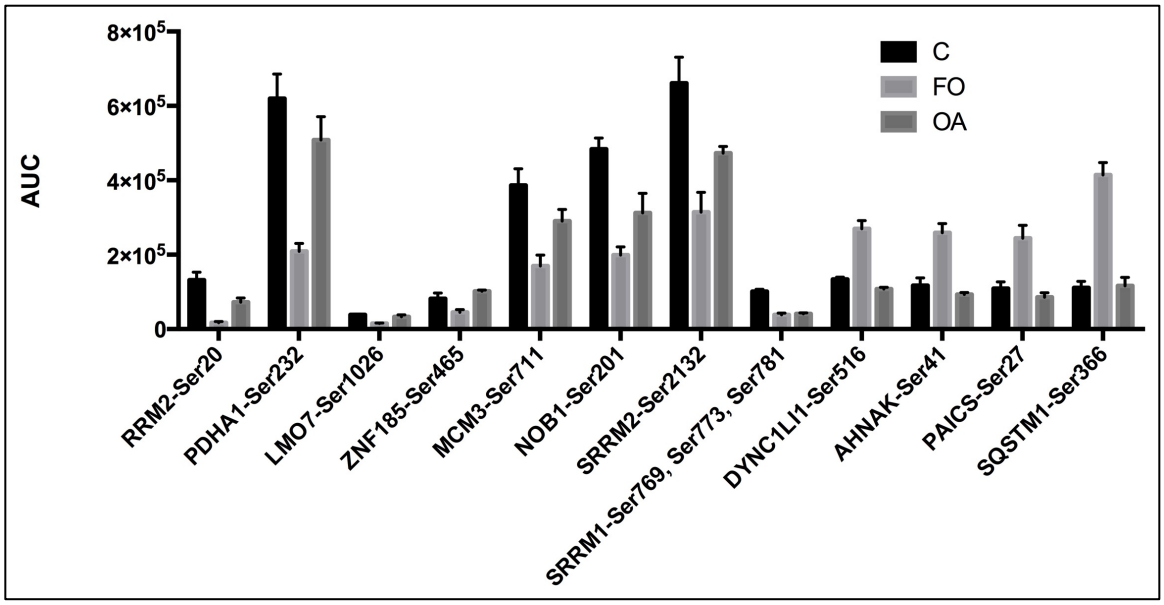


Figure 4-6 Relative abundance of the significantly changed phosphoproteins under different treatment conditions.

The relative abundance levels were calculated based on the phosphopeptide intensities with fish oil (FO)-treated, oleic acid (OA)-treated or control (C). (n=4)

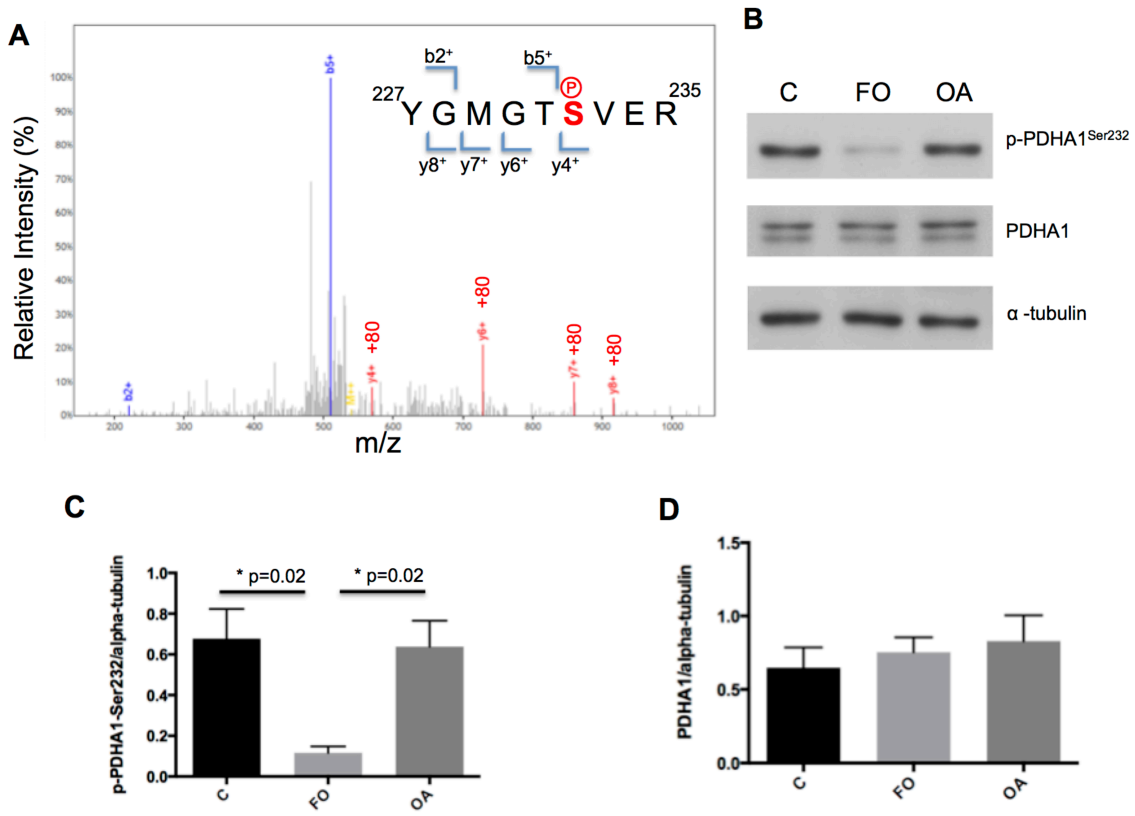


Figure 4-7 Phosphoprotein validation of phospho-PDHA1^{S232}.

A: MS/MS spectrum of phosphopeptide ^{227}YGMGTSVER^{235} from PDHA1^{S232}; **B**: Western blot of phospho-PDHA1^{S232}, total PDHA1, and α-tubulin, which served as a loading control; **C**: Bar graph of relative intensity of phospho-PDHA1^{S232} to a-tubulin, n=3 from Western blot of phospho-PDHA-1^{S232} (B); **D:** Bar graph of relative intensity of total PDHA1 to a-tubulin, n=3 from Western blot of PDHA1 (B).

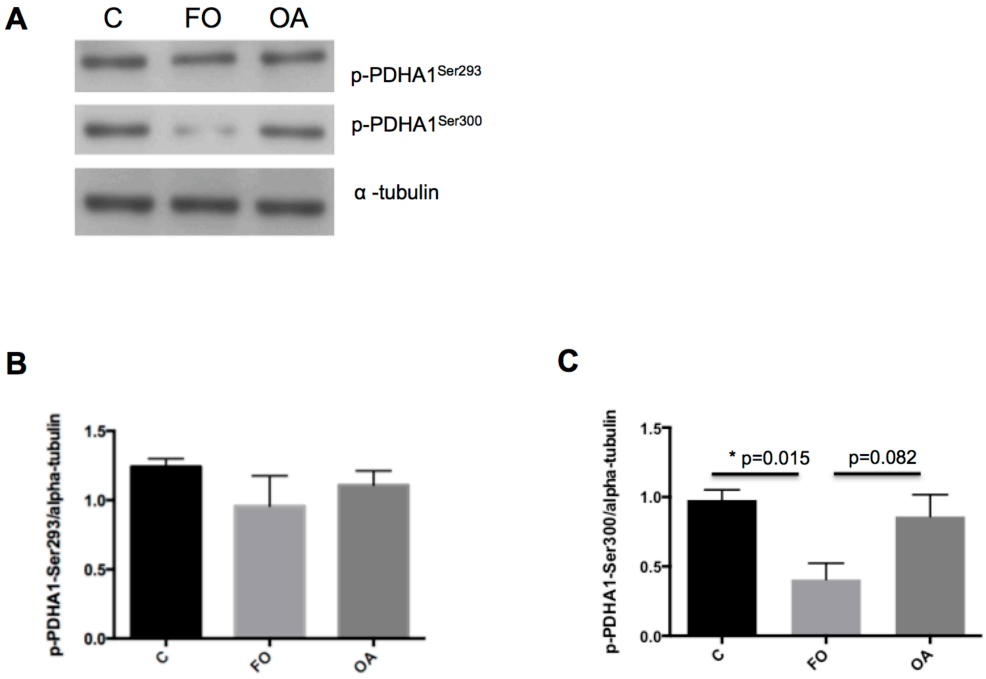


Figure 4-8 Phosphoprotein phospho-PDHA1^{S293} and phospho-PDHA1^{S300}.

A: Western blot of phospho-PDHA1^{S293}, phospho-PDHA1^{S300} and α-tubulin, which served as a loading control; **B**: Bar graph of relative intensity of phospho-PDHA1^{S293} to α-tubulin, n=3 from Western blot of phospho-PDHA1^{S293} (A); **C**: Bar graph of relative intensity of phospho-PDHA1^{S300} to α-tubulin, n=3 from Western blot of phospho-PDHA1^{S300} (A)

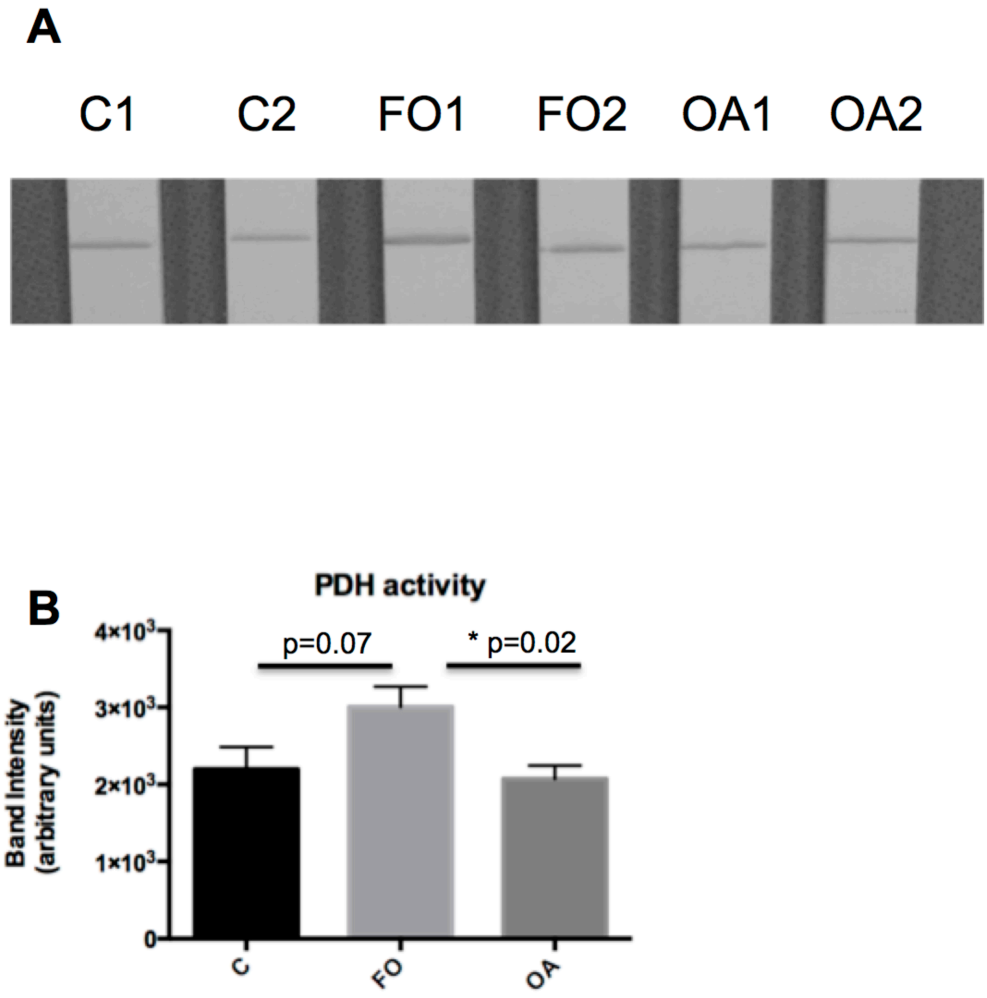


Figure 4-9 PDH activity measurement.

A: PDH activities measured by a dipstick assay kit; **B:** Quantitative bar graph of PDH activities from the dipstick assay (A), n=5.

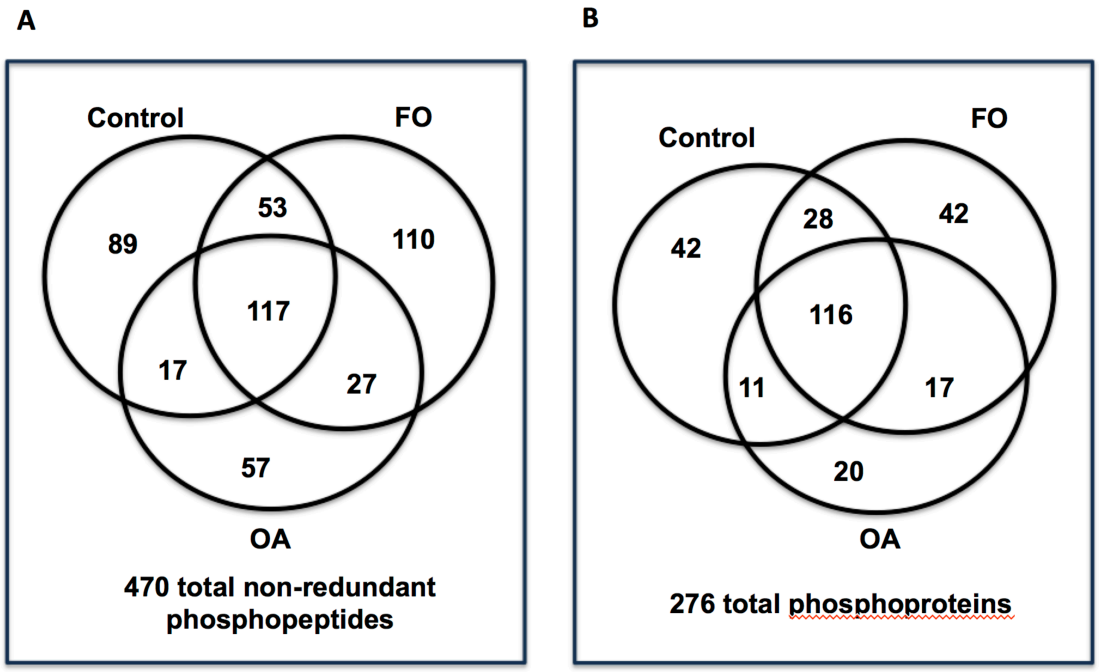


Figure 4-10 Venn diagrams of identified phosphopeptides and corresponding phosphoproteins in day-6 FAs treatment.

A: Number of non-redundant phosphopeptide identified in at least 1 of 4 replicates under each condition. **B**: Number of corresponding phosphoproteins.

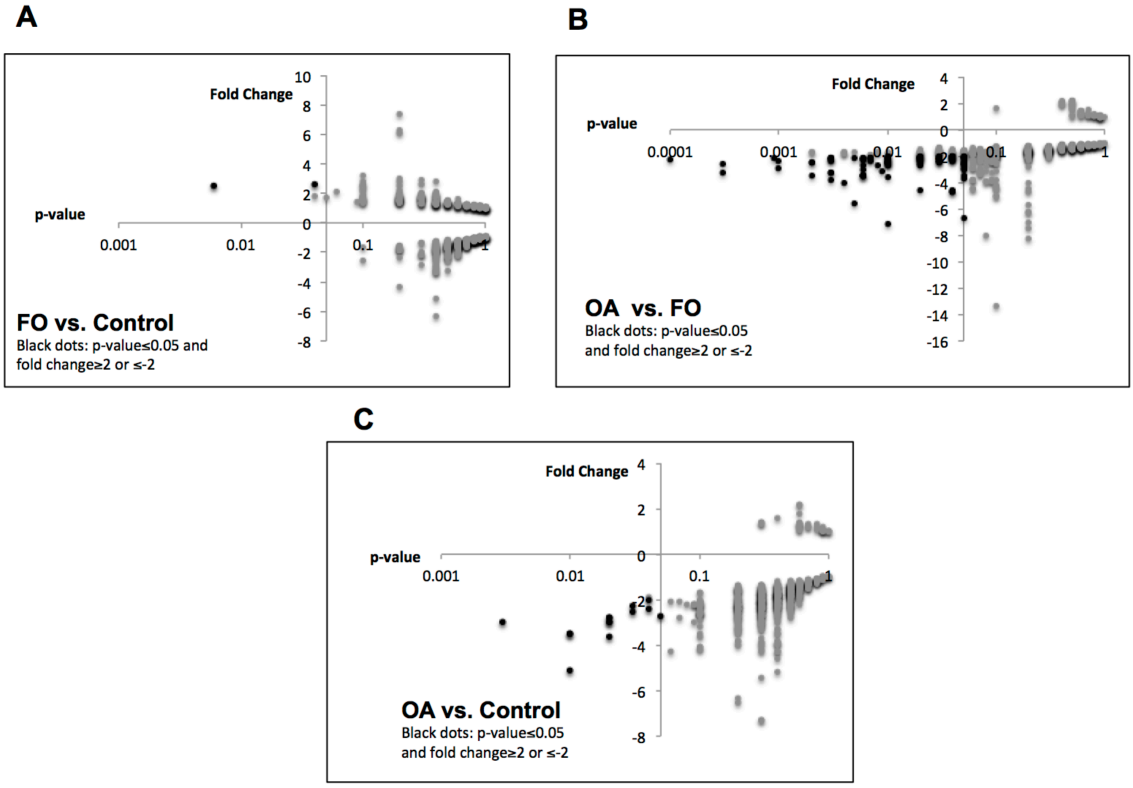


Figure 4-11 Relative comparisons of phosphopeptide level based on label-free quantification in day-6 FAs treatment.

Fold change (y-axis) and t-test p-value (x-axis) were presented for each phosphopeptide (n=518). P-value axis crosses fold change axis at p=0.05. Relative changes with p-value≤0.05 and fold change≥2 or ≤-2 were labeled in each figure by black dots (n=4).

Chapter 5 Conclusions and future perspectives

Previous studies on the effects of PUFAs on prostate cancer showed contradictory outcomes [98, 105], which make it difficult to classify certain dietary FAs as protective or detrimental supplements. Some studies were considered to have questionable experimental design [243-245]. This is further complicated by the heterogeneity of dietary FAs intake and corresponding FA composition in human bodies [246]. Dietary FAs can be subdivided into saturated and unsaturated FAs. Among unsaturated FAs, omega-3, -6 or -9, and FAs with different length of carbon chain, have divergent functions. Different kinds of food ingredients contain different FAs and different combinations of various FAs. According to a systematic review of 37 studies, conclusions are inconsistent and unclear [119]. Studies carried out involve different FA measurements strategies, different populations and different endpoints of measurement. Most indications of PCa incidence are based on histological confirmation or medical record [96, 247]. Population of participants in these studies varies from hundreds [248] to thousands [249] and length of fish/fish oil consumption ranges from weeks [99] to years [250]. These studies also involved different FA measurement strategies. One of the publications, which failed to support n-3 FAs' protective role measured serum-derived phospholipid FAs and the data was presented as percentage of each FA in total phospholipid FAs [105], while other studies measured FA composition in red blood cells [251] or prostatic tissues [98]. Many other papers, instead of evaluating body FAs levels, looked for correlation between fish consumption and PCa incidence [250, 252]. Some studies find that specific FAs rather than all FAs impact PCa risk. For example, EPA [98]

and DHA [96] are reported to reduce PCa risk. However, these studies also evaluated different endpoints. According to a recent review [119], some studies measured PCa incidence as an endpoint of omega-3 PUFA's impact on PCa [96] but some other studies measured PCa aggressiveness [250]. The contradictory conclusions suggest that FO may regulate many other undiscovered pathways and molecules, which may help explain the complexity of FO's effects.

Compared to many other drugs, which have specific identified targets, FAs regulate cell function in a multi-target and multi-level fashion. Previous studies found FO may exert its function by inducing survival stress [121], changing glucose metabolism [202], altering plasma membrane composition [253] or modulating inflammatory factors [254]. Our study identified several potential novel drug targets and pathways associated with FO action.

In the global proteomic study, only very modest changes were discovered with FO treatment even with significant changes in cancer cell viability. A possible explanation can be that in cell culture, PCa cells are exposed to abundant nutrients, which can be very different from the limited nutrients in the *in vivo* environment in which cancer grows. In addition, FO consumed through the diet may be catabolized *in vivo* into additional compounds, which does not occur in an *in vitro* environment. Some of the biologically important changes would need to be validated by other biochemical and/or biophysical methods.

1 FO induces survival-threatening stress in cancer cells

N-3 PUFAs are reported to induce cytotoxicity by triggering reactive oxygen species (ROS) [121, 255, 256]. Our study confirmed that FO can inhibit viability in PC3 cells within 24-hours after treatment. However, the overall proteome profile under short-term FO treatment condition (one day) did not change significantly as compared to prolonged treatment (i.e., six days). After candidate selection using stringent criteria, the most significantly changed protein was sequestosome-1, ~1.7-fold overexpression induced by FO. One reasonable assumption is that the increased level of sequestosome-1 expression is the result of autophagy inhibition [189]. However, our follow-up study on autophagy showed that FO induced little change in LC3 II, biomarker of autophagy, indicating that FO had no direct effect on autophagy. Our observation is not in agreement with a previously published study in which the authors suggested that DHA induces not only apoptosis but also autophagy [256]. Combined with our q-PCR results, which suggested that FO elevated sequestosome-1 expression at the transcriptional level, we hypothesize that FO-triggered stress induces accumulation of misfolded proteins and the accumulation rate exceeds the product elimination rate. Thus the FO treated cells need to synthesize more sequestosome-1, which directs the protein to be degraded via lysosomes. Our study suggests the *de novo* synthesis instead of degradation inhibition is a more likely explanation for the accumulation of sequestosome-1 with FO treatment. Consistent with this, a report studying ovarian cancer chemo-resistance discovered that sequestosome-1 is upregulated in a drug-resistant cell line and relieved ROS stress induced by H_2O_2 [257]. From the phosphoproteome study, it was suggested that phospho-serine366 of sequestosome-1 was upregulated to as large as 3.7-fold. Ser-366 is

located in the PEST domain, which is believed to be associated with degradation [217]. However, we cannot conclude whether the induction of phosphorylation is due to the total protein level elevation, site-specific phosphorylation increase, or combination of both, given that the total protein level is up-regulated. Considering that sequestosome-1 was up-regulated at the transcriptional level, the protein over-expression is not the result of phosphorylation-dependent degradation changes. Some proteins that regulate DNA synthesis or cell cycle, such as phospho-RRM2 and phospho-MCM3, also exhibited significant changes under FO treatment. RRM2 is responsible for deoxyribonucleotide synthesis and FO significantly decreased $RRM2^{S20}$ phosphorylation levels, which has been reported to be associated with Wnt pathway [222]. Through phosphorylating $RRM2^{S20}$, Wnt3a can rescue the Wnt pathway from inhibition by RRM2. Considering Wnt is one of the oncogenic pathways [258], our discovery that FO dephosphorylates $RRM2^{S20}$ is consistent with the protective role of FO. MCM3 is one of the proteins that control the cell cycle checkpoint and $MCM3^{S711}$ phosphorylated by cyclin E/Cdk2 is down-regulated by FO [223, 224]. Taken together, when PC3 cells are treated with FO, the first wave of stress stimulated responses may include 1) synthesizing more sequestosome-1 to eliminate misfolded/unfolded proteins and 2) regulating phosphorylation level of cell cycle related proteins, such as RRM2 and MCM3, to block cell proliferation.

2 FO influences the protein levels of glycolytic enzymes

FAs are major components of living organisms. Treatment with FAs lead to changes in default bioenergy regulation and metabolism. It is clear that when extra FAs

are added into the medium in our experiments, the *de novo* synthesis pathway will be suppressed. However, from our experimental results, FAs not only altered lipid but also glucose metabolism, even in PCa, in which glucose is not a main energy source. The up-regulation of aerobic glycolysis is considered as a hallmark in the progression of most tumors, and this phenomenon is known as the Warburg effect [259]. Our global proteome profile indicates several glycolysis/glycogenesis enzymes, including GPI, GAPDH, PGK1 and PK, are down-regulated after prolonged (6 days) FO treatment. In addition to glycolysis processes, these proteins also play roles in other bioprocesses.

GPI catalyzes the conversion of glucose-6-phosphate to fructose-6-phosphate. GPI is also known as autocrine motility factor (AMF). Besides its role in glycolysis, GPI/AMF is a secreted cytokine and plays a critical part in metastatic cancers [260, 261]. GPI/AMF can promote tumor growth and suppress apoptosis through activating PI3K/AKT and ERK pathways [262]. The expression of GPI/AMF has been found to be elevated in endometrial carcinoma [263]. In patients with clear cell renal cell carcinoma, highly expressed GPI/AMF is positively correlated with poor prognosis [264].

GAPDH catalyzes the conversion of glyceraldehyde-3-phosphate to 1,3 bisphospho-glycerate. GAPDH is a house keeping protein and is used as an internal loading control for Western blotting in many studies. However, its expression can be affected in some specific circumstances, such as with FO treatment in this study [265]. Recent studies suggest that, like GPI, GAPDH is also a moonlighting protein with bioactivities other than the glycolytic role [266]. GAPDH participates in the fusion of vesicles [267] and organization of the cellular skeleton [268]. GAPDH is also discovered to modulate stabilization of mRNAs of some genes by binding to AU-rich elements in 3'-

untranslated regions [269, 270]. In one prostate cancer study, GAPDH is shown to be a co-activator in AR dependent transactivation and this function is irrelevant to glycolysis [271].

PGK1 catalyzes 1,3 bisphosphate-glycerate to form glycerate-3-phosphate. Prostate cancer cells are reported to secret PGK1 to regulate bone formation at bone metastatic sites, by inducing osteoblastic differentiation and inhibiting osteoclastogenesis [272]. PGK1 secreted by stromal cells has also been shown to can promote prostate cancer growth and invasiveness [273].

Pyruvate kinase was one of the proteins down-regulated and is involved in the transfer of phosphate groups from phosphoenolpyruvate to ADP to produce pyruvate and ATP. High expression of PKM2 is observed in more advanced prostate cancer [274]. Interestingly, another paper pointed out the nuclear translocation of PKM2 may induce epithelial-mesenchymal transition and promote metastasis of prostate cancer [275].

Our results suggest that expression levels of some glycolytic enzymes are suppressed by FO. However, these changes are small but statistically significant. Furthermore, considering we did not evaluate the aerobic glycolysis rate, we cannot conclude that FO inhibits glycolysis in prostate cancer. Further metabolic studies are needed to address this. Some of these enzymes have additional functions other than serving in glycolysis and some of these functions are oncogenic [263, 271, 273, 275]. Whether FO is capable of influencing the microenvironment distribution or subcellular localization of these enzymes would also be interesting to look into.

While these protein level changes of glycolytic enzymes take place after prolonged treatment (six-days) of FO, shorter incubation with FO (one-day) also triggers

changes related to glucose oxidation. Pyruvate can be broken down into acetyl-CoA by PDH enzymes, which link the glycolysis and the TCA cycle. In turn, PDH is regulated by pyruvate dehydrogenase kinases (PDK) through phosphorylation and inactivation, and pyruvate dehydrogenase phosphatase through dephosphorylation and activation. In our phospho-proteome study, after the first day of FO treatment, phospho-pyruvate dehydrogenase A1 (phospho-PDHA1^{S232}) was down-regulated, while PDH activity was found to be increased. Meanwhile in this phosphoproteome study, for the other two phosphorylated sites, phospho-PDHA-1^{S293} is also down-regulated in FO group compared to control (fold change = -1.3, p=0.03) though the change is not significant when analyzed by Western blot while phospho-PDHA-1^{S300} had not been identified in the phosphoproteome study but was found suppressed in Western blot analysis. The combination results of the proteome and phospho-proteome studies implicate that FO treatment may suppress the glycolytic pathway by restoring the connection between the glycolysis product, pyruvate, with TCA cycle through dephosphorylating PDHA1 at serine-232 and serine-300 and elevating the enzyme activity in short-term treatment while suppressing glycolysis enzyme expression level after long-term treatment.

3 FO alters plasma membranes, intracellular trafficking and cytoskeleton

Dietary FA intake changes the composition of plasma membranes [253]. There are two aspects it may affect. First, it may alter the microenvironment by affecting the lipid raft holding the membrane attaching proteins, many of which are key cell signal transducing molecules [276, 277]. Second, based on our study, it may change the normal cargo trafficking by affecting the intracellular vehicle membrane and the

associated proteins. Our phosphoproteome study found that FO changes phosphorylation of phospho-DYNC1LI1 (cytoplasmic dynein 1 light intermediate chain 1), a protein involved in intracellular transportation [278]. It is worth noting, according to our day-6 FO treatment global proteomic study results, many cell skeleton proteins, such as tubulin (including tubulin beta-3 chain, fold change = -1.2, p=0.01; tubulin alpha-4A chain, fold change= -1.2, p=0.07), vimentin (fold change = -1.2, p=0.03) and keratin (fold change = -1.2, p=0.05), are all down-regulated by FO.

4 FO alters pro-inflammatory pathways

N-3 PUFAs are considered to be anti-inflammatory and anti-oncogenic [279-281]. Besides known anti-inflammatory metabolites, such as PGE3 or LTB5 [282], our study also reveals its suppressive function on a recently identified inflammatory molecule MSMP (prostate associated microseminoprotein), which is a strong ligand for CCR2B [207]. This discovery not only gives new insight into FO's anti-inflammatory effect but also suggest that MSMP might be a potential biomarker to monitor FO's effect on PCa. In addition, as MSMP is a secreted protein, it might be able to be detected in body fluid, such as plasma or prostate secretion and serve as a novel biomarker for detection of primary PCa or disease relapse. Follow-up studies could be carried out to measure MSMP levels in plasma or urine of patients with different PCa stage and clinical trials can also be performed to check whether MSMP levels are changed before and after treatment.

5 FASN expression may regulate pro-inflammatory prostaglandin production

FAs produce numerous bioactive metabolites including prostaglandins, thromboxanes and leukotrienes through enzymes such as LOXs and COXs [283]. Some of these metabolites are pro-inflammation/pro-oncogenic and others may have opposing functions [284, 285]. Previous studies showed that metabolites of n-6 PUFAs are more inflammatory compared to those of n-3 PUFAs. What adds to the complexity is that in different environments the enzyme expression and activity may differ [150, 286]. In addition to this, these enzymes can be regulated by different FAs similar to that of COX-2 expression altered by increased n-3/n-6 PUFA ratios [158]. Our studies find a regulatory connection between COX-2 and FASN that COX-2 expression is decreased when FASN is depleted. Considering that the only known function of FASN is synthesizing palmitate, whether the regulation is direct or indirect still needs additional investigation. As both FASN and COX-2 are overexpressed in cancer cells [53, 150], are both related to inflammation and down-regulated by n-3 PUFAs, the discovery that FASN protein depletion can lead to COX2 decrease may render another explanation for FO anti-inflammatory effects.

The FASN siRNA knockdown study also explored additional potential targets of FASN. The purpose and concept of studying the pathways modulated by FASN is similar to the FA study, with the hypothesis that FASN knockdown would dramatically change pathways modulated by fatty acid synthesis products. In our study of FASN in PC3, there is only a partial suppression of pathway activity by FO although the expression change is significant. This may lead to limited downstream target changes.

The outcome of our study suggests that even when total protein levels are suppressed, some functional residual FASN proteins may remain active.

6 Limitations and future directions

Like many studies, there are limitations to our studies. 1) Proteins with critical roles may not be detected due to their low abundance, which is beyond the detection limit of the mass spectrometry. 2) Many proteins we identified have changes that are statistically significant but have small changes. In addition to the assumption we made that expressions of certain proteins are changed, it is also possible that FO exerts its function through other mechanisms. Besides the probable alterations on PTM, it is also possible that the bioactive metabolites of FO change the biological processes in the cells. Many metabolites of FO, such as PGE3, 15-HEPE and LTB5 are proved to be anti-inflammatory or anti-mitogenic [282, 287, 288]. 3) The functions of the majority of the phosphorylation sites are not well characterized, which limits the ability to analyze the causative factors. 4) The molecular targets and pathways regulated by FO are only identified in one PCa cell line, PC3. To find out if these changes are universal and cancer-specific, it is necessary to validate these results in other prostate cancer cell lines, benign prostate cells and cell lines from other cancer types. 5) This study was carried out only in *in vitro* conditions. *In vivo* studies are needed to validate the discoveries made in these studies, i.e., in animal models and clinical trials.

To conclude, our studies investigated both global proteome changes and global phosphoproteome changes modulated by FO and OA. Cross comparison of the two proteome-wide studies and follow-up protein expression/function verification

experiments confirmed the potential targets associated with the PUFA treatment and that were specific to FO treatment. In addition, the analysis of the time-course study dissected out complex regulatory pathways into less complicated step-wise functions altered by FO over time. This study confirmed that FO changed PCa cell function through diverse pathways including glycolysis, cell cycle, cytotoxicity induced stress, anti-inflammation and also provided useful details about the mechanism of these effects by identifying novel protein and phosphoprotein targets such as MSMP, phospho-sequestosome^{S366} and phospho-PDHA1$^{S232, S300}$ **(Fig. 5-1)**. This study also discovered an interesting regulatory connection between FASN and COX2. Considering the success of the combination of global proteomic and phosphoproteomic studies to identify new important proteins altered by FA treatment in PC3 cells, a global proteome and phosphoproteome clinical study in PCa patients would validate and extend these findings to the patient population and potentially identify additional targets of FO action, providing novel opportunities for therapeutic development.

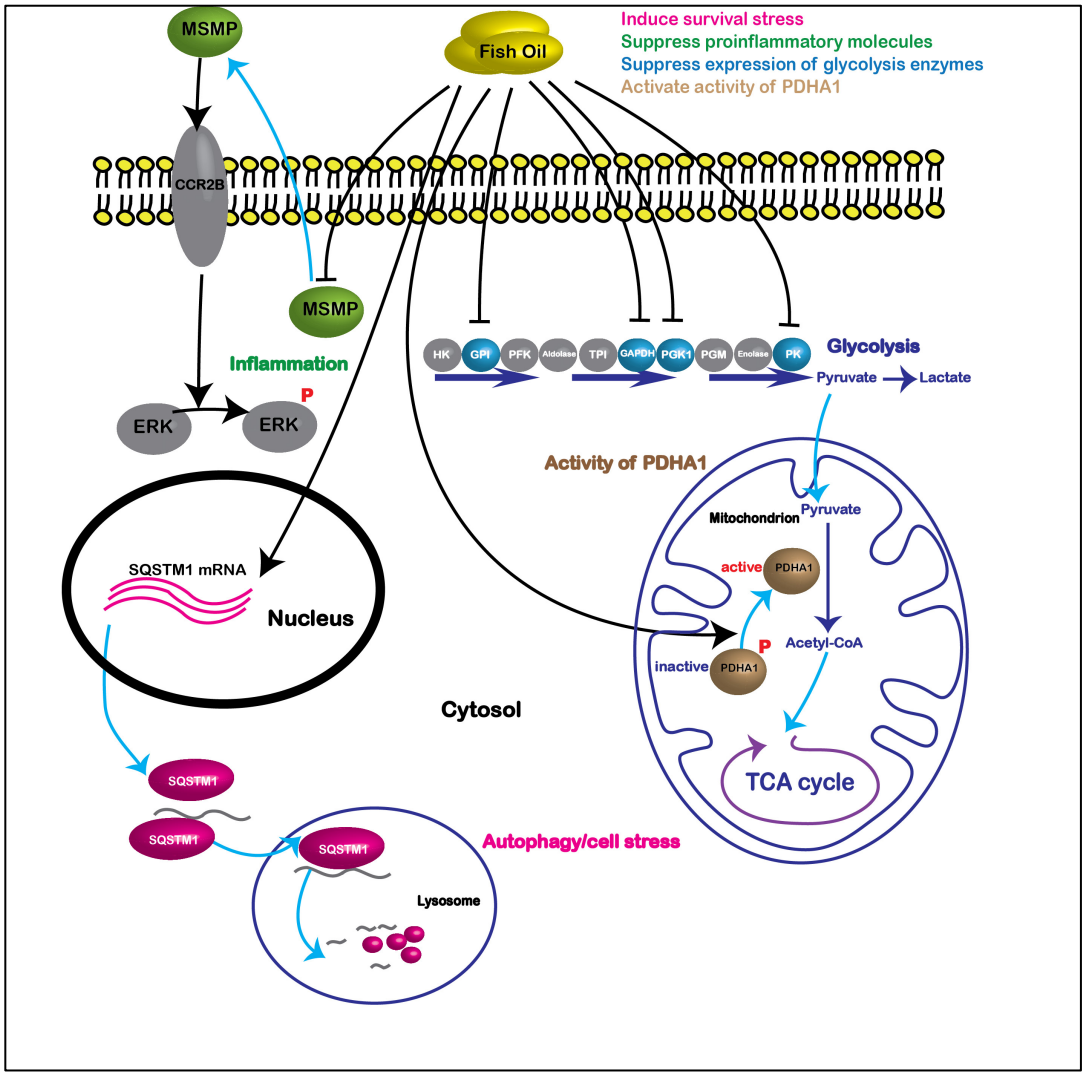


Figure 5-1 Multiple pathways in PC3 cell line are regulated by fish oil treatment.

Multiple proteins involved in multiple biological processes including cytotoxicity induction, inflammation suppression, and glycolysis inhibition, PDH activation were altered after the fish oil treatment.

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